The effects of metformin on metabolism and cardiovascular disease in type 2 diabetes

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The effects of metformin on metabolism and cardiovascular disease in type 2 diabetes

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Chapter 1

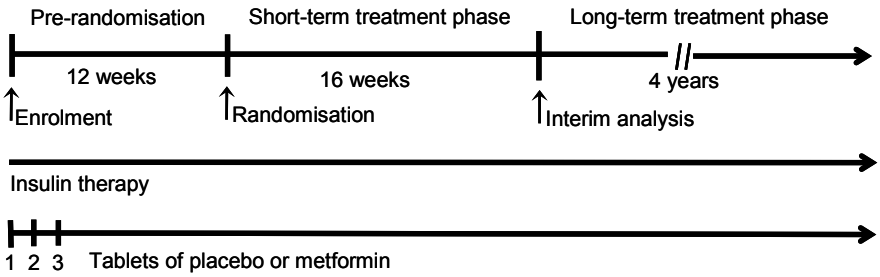
General introduction

Introduction

The incidence of type 2 diabetes is rising. The number of adults older than 20 years of age having diabetes worldwide in the year 2000 is estimated to be ~171 million and is anticipated to more than double before 2030.[1] Up to 75% of patients with type 2 diabetes will die of a cardiovascular complication,[2] making the prevention of cardiovascular disease in diabetes an important therapeutic target.

The first randomised intervention trial on this subject was the UKPDS,[3] the results of which suggest a cardioprotective role for metformin. However, the design and analyses of the UKPDS have raised considerable debate, leaving a need for additional clinical, randomised, intervention trials. In addition, the mechanisms through which metformin might influence cardiovascular disease are not fully elucidated. These questions inspired the design of the randomised, placebo-controlled, multicenter trial "Hyperinsulinaemia: the Outcome of its Metabolic Effects" (HOME) to investigate these issues in 1997.

Figure 1 HOME trial profile



Metformin

Metformin is an oral anti-diabetic drug from the biguanide class. It was first described in the scientific literature in 1957 and first marketed in France in 1979, but only received approval for treatment in type 2 diabetes by the U.S. Food and Drug Administration in 1994.

Antihyperglycaemic action

Metformin lowers glucose mainly by reducing hepatic glucose output through inhibition of gluconeogenesis, and to a lesser extent, glycogenolysis. In addition, metformin might increase glucose uptake in skeletal muscle and adipocytes,[4] and increases insulin sensitivity.[5] Metformin may increase the glucose transport capacity of glucose

transporters and facilitate trafficking of glucose transporters to the plasma membrane.[4] The exact mechanism through which metformin reduces hepatic glucose production is not entirely clear, but activation of AMP-activated protein kinase (AMPK), a major cellular regulator of glucose and lipid metabolism, which is required for inhibition of hepatocyte glucose production, seems to play an important role.[6] Increased peripheral utilisation of glucose may be due to improved insulin binding to insulin receptors,[4] in which AMPK may also play a role, as metformin administration increases AMPK activity in skeletal muscle.[7] Other data show, however, that metformin is able to alter glucose utilisation even when AMPK activation is inhibited, suggesting that some of the metabolic actions of metformin might occur independently of AMPK activation.[8]

Other metabolic actions

Recent research also points to AMPK as an important regulator in other pathophysiological processes, such as dyslipidaemia, regulation of weight, endothelial dysfunction, and low-grade inflammation.[5-7, 9] AMPK activation increases hepatic fatty acid oxidation and inhibits the proximal and rate-limiting step of lipogenesis and is thereby likely to influence triglyceride and LDL cholesterol levels. AMPK might also mediate the effects of metformin on body weight. Treatment with both AICAR, a chemical activator of AMPK, and metformin decreases fat mass.[10] In addition, ACC knockout mice, lacking acetyl-CoA carboxylase and thereby mimicking AMPK activation, maintain or lose weight despite increased food intake.[11] Finally, studies suggest that metformin inhibits nuclear factor kappaB activation through AMPK activation, thereby inhibiting NF-kappaB-dependent gene expression of various inflammatory and cell adhesion molecules, important in endothelial (dys)function and low-grade inflammation.[9, 12]

Metformin and cardiovascular disease

Glycaemic control and hyperinsulinaemia

In type 2 diabetes, epidemiological studies have shown strong associations between the degree of hyperglycaemia and the incidence of cardiovascular disease.[13] However, contrary to microvascular disease, interventional studies have yet failed to demonstrate that lowering glucose levels unequivocally results in a reduction in the incidence of cardiovascular disease or death.[14] Epidemiological studies have shown hyperinsulinaemia to be an independent risk factor for cardiovascular disease,[15-17] but no interventional data on this issue exists. Short-term studies have shown that metformin improves glycaemic control and reduces insulin requirements.[18-20]

Other traditional risk factors

Besides hyperglycaemia and hyperinsulinaemia, other traditional risk factors such as weight gain, dyslipidaemia, and blood pressure have been shown to be strong predictors of cardiovascular disease.[21, 22] In interventional trials, improving dyslipidaemia and reducing blood pressure improve cardiovascular outcome.[23-26] Interventional studies on weight loss and its influence on cardiovascular outcome are ongoing.[27] Short-term

studies show that metformin prevents weight gain[18-20] and may moderately improve dyslipidaemia.[28-30] However, evidence suggests that metformin has no intrinsic effect on blood pressure.[28, 29, 31, 32]

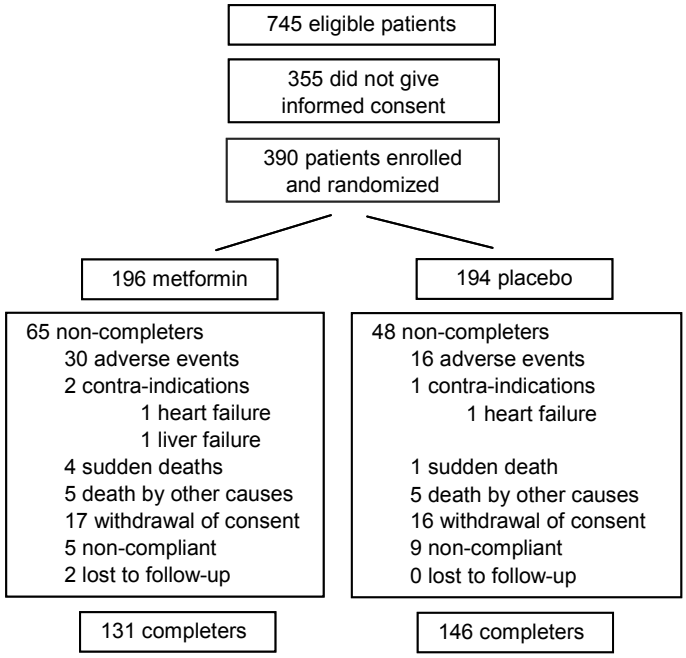
Endothelial dysfunction and low-grade inflammation

Although traditional risk factors have been shown to predict cardiovascular disease, epidemiological studies have also shown that they can only partly explain the increased risk of cardiovascular disease in type 2 diabetes.[22, 33] Observational studies have found strong associations between markers of endothelial dysfunction and low-grade inflammation and cardiovascular disease.[34, 35] There is some evidence that metformin may improve these markers,[36-39] but most focus on markers of fibrinolysis only,[37-39] which may or may not reflect endothelial function.[40]

Vitamin B12, folate, and homocysteine

There are few disadvantages to the use of metformin. However, metformin induces vitamin B12 malabsorption, which may increase the risk of developing vitamin B12 deficiency.[41-43] In addition, metformin treatment has been reported to be associated with decreased folate concentrations, although the mechanism of this effect has not been elucidated.[43] Both decreases in folate and vitamin B12 levels might, in turn, result in an increase in homocysteine levels, an independent risk factor for cardiovascular disease, especially among individuals with type 2 diabetes.[44-46]

Figure 2 HOME trial flow chart



Aims of this thesis

This thesis describes the results of a randomised, placebo-controlled trial in type 2 diabetic patients treated with insulin, the HOME trial. In 1997 the HOME trial was designed to study the metabolic and (cardio)vascular effects of metformin in a setting of similar glycaemic control, but different levels of insulinaemia, between two treatment groups during a follow-up of 4.3 years.[20] Figure 1 and 2 describe the HOME trial design in more detail. In addition, epidemiological data from the Hoorn Study, a prospective population based cohort study of glucose tolerance and cardiovascular disease, are reported in this thesis.[47, 48]

The following research questions are addressed in subsequent chapters:

1. To what extent can the association of type 2 diabetes with cardiovascular disease be accounted for by endothelial dysfunction and low-grade inflammation? Is this association of endothelial dysfunction and low-grade inflammation on the one hand and cardiovascular disease on the other independent of other conventional cardiovascular risk factors and indicators, i.e. do these pathophysiological mechanisms largely overlap or represent distinct pathways of disease? We studied this issue (and included the results in this thesis) to provide insight on whether or not to study the effects of metformin, in the HOME trial, on markers of endothelial dysfunction and low-grade inflammation (see below).
   Chapter 2: Endothelial dysfunction and low-grade inflammation explain much of the excess cardiovascular mortality in individuals with type 2 diabetes – the Hoorn Study

2. Does short-term treatment with metformin improve endothelial function and decrease inflammatory activity? What biochemical mechanisms might mediate such a metformin-associated improvement, if any?
   Chapter 3: Effects of short-term treatment with metformin on markers of endothelial function and inflammatory activity in type 2 diabetes mellitus: a randomised, placebo-controlled trial

3. Will long-term treatment with metformin have sustained beneficial metabolic effects, even at the same level of glycaemic control, and thus decrease cardiovascular disease?
   Chapter 4: Long-term effects of metformin on metabolism and micro- and macrovascular disease in patients with type 2 diabetes

4. Does long-term treatment with metformin improve endothelial function and decrease inflammatory activity, and thereby decrease the risk of cardiovascular disease?

Chapter 5: Metformin improves markers of endothelial function, but not inflammation in type 2 diabetes treated with insulin: a long-term, randomised, placebo-controlled trial

5. Does long-term treatment with metformin lead to decreased levels of vitamin B12, or even vitamin B12 deficiency, decreased levels of folate, and increased levels of homocysteine?
   Chapter 6: Long-term treatment with metformin in patients with type 2 diabetes treated with insulin is associated with an increased risk of clinical vitamin B12 deficiency, and possibly with an increase in homocysteine levels: a randomised, placebo-controlled trial

The final chapter (Chapter 7) discusses the present findings, limitations and implications of the study, and offers a perspective to future research.

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Chapter 2

Endothelial dysfunction and low-grade inflammation explain much of the excess cardiovascular mortality in individuals with type 2 diabetes: the Hoorn Study

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Abstract

Objective

The mechanisms responsible for the increased cardiovascular disease risk that accompanies type 2 diabetes (T2D) remain poorly understood. It is commonly held that endothelial dysfunction and low-grade inflammation can explain, at least in part, why deteriorating glucose tolerance is associated with cardiovascular disease. However, there is no direct evidence for this contention.

Methods and Results

In this population-based study (n=631), T2D was cross-sectionally associated with both endothelial dysfunction and low-grade inflammation, whereas impaired glucose metabolism (IGM) was associated only with low-grade inflammation. These findings were independent of other risk factors that accompany T2D or IGM. During a follow-up of 11.7 years (median; range 0.5 – 13.2 years), low-grade inflammation was associated with a greater risk of cardiovascular mortality (hazard ratio, 1.43 [95% CI, 1.17 to 1.77] per 1 SD difference). For endothelial dysfunction, the association with cardiovascular mortality was stronger in diabetic (hazard ratio, 1.87 [1.43 to 2.45]) than in non-diabetic individuals (hazard ratio, 1.23 [0.86 to 1.75]; P-interaction = 0.06). Finally, T2D-associated endothelial dysfunction and low-grade inflammation explained about 43% of the increase in cardiovascular mortality risk conferred by T2D.

Conclusions

These data emphasize the necessity of randomised controlled trials of strategies that aim to decrease cardiovascular disease risk by improving endothelial function and decreasing low-grade inflammation, especially for T2D patients.

Introduction

Up to 75% of individuals with type 2 diabetes (T2D) will die of cardiovascular disease.[1] The mechanisms responsible for the high cardiovascular disease risk that accompanies T2D and, possibly, impaired glucose metabolism (IGM; i.e. impaired fasting glucose and (or) impaired glucose tolerance),[2] however, remain poorly understood. There is strong evidence that conventional risk factors, such as hypertension, obesity and dyslipidaemia, cannot fully explain the high cardiovascular disease risk associated with deteriorated glucose tolerance.[3]

It is commonly held that endothelial dysfunction and low-grade inflammation, two key features in the pathophysiology of atherothrombosis,[4-7] can explain, at least in part, why deteriorated glucose tolerance is associated with cardiovascular disease.[2,8] However, there is no direct evidence for this contention and several important issues have remained unresolved. First, it is not clear to what extent the associations of IGM and T2D on the one hand with endothelial dysfunction and low-grade inflammation on the other are independent of other risk factors associated with deteriorated glucose tolerance. Second, it is not known whether associations of endothelial dysfunction and low-grade inflammation with cardiovascular disease are independent of other conventional cardiovascular risk factors and indicators, nor to what extent these associations overlap or represent distinct pathways. If these pathways are distinct, then associations of endothelial dysfunction and low-grade inflammation with cardiovascular disease will be expected to be mutually independent. Finally, it is not known to what extent associations of IGM and T2D with cardiovascular disease are in fact accounted for by IGM- and T2D-associated endothelial dysfunction and low-grade inflammation.

We addressed these questions in the Hoorn Study, a prospective population-based cohort study of glucose tolerance and cardiovascular disease.[9,10]

Research design and methods

General Study Design

This study was part of the Hoorn Study, a population-based cohort study of glucose tolerance and cardiovascular disease in a Caucasian population in Hoorn, the Netherlands, of which the baseline measurement was performed from October 1989 to February 1992.[9,10] Briefly, a random sample of all men and women aged 50-75 was drawn from the municipal population registration office of Hoorn; 2484 individuals participated (response rate 71%). The present study population is an age-, sex-, and glucose-tolerance-stratified random subsample (n=631; response rate 89%), in whom an extensive investigation of diabetes complications was performed.[9,10] For the present analyses, we used the 1999 WHO criteria and classified individuals as having normal glucose metabolism (NGM), impaired glucose metabolism (IGM), or type 2 diabetes mellitus (T2D) based on two glucose tolerance tests.[11] The Hoorn Study was approved

by the Ethical Review Committee of the VU University Hospital. Written informed consent was obtained from all participants.

Baseline investigations

We considered plasma levels of von Willebrand factor (vWf) and soluble vascular adhesion molecule-1 (sVCAM-1) as markers of endothelial function,[12-14] and plasma levels of C-reactive protein (CRP) and soluble intercellular adhesion molecule-1 (sICAM-1) as markers of low-grade inflammation.[15,16] Microalbuminuria was not used as a marker of endothelial function, because we have previously shown that microalbuminuria in the Hoorn Study is heterogeneous in terms of its association with endothelial dysfunction.[17]

Markers of endothelial dysfunction and low-grade inflammation

Concentrations of vWf, sVCAM-1, CRP, and sICAM-1 were assessed in deep frozen (-70˚C) heparin plasma samples. vWf, sVCAM-1, and sICAM-1 were estimated in duplicate by ELISA.[18-20] For vWf and sVCAM-1 no plasma samples were available for 21 subjects. Concentrations of CRP were measured with a highly sensitive sandwich enzyme immunoassay, as described before.[18] For CRP and sICAM-1 no plasma samples were available for 23 subjects.

Other measurements

We obtained an ankle-brachial blood pressure index (n=631) and a resting electrocardiogram (n=625).[9,10] Subjects were classified as having cardiovascular disease when they had a history of myocardial infarction and/or had an electrocardiogram with a Minnesota code 1.1-1.3, 4.1-4.3, 5.1-5.3, or 7.1 and/or had undergone coronary bypass surgery or angioplasty, and/or had an ankle-brachial pressure index less than 0.9 in either leg and/or had undergone a peripheral arterial bypass or non-traumatic amputation. In addition, we obtained data on blood pressure, weight, height, body mass index, waist-to-hip ratio, glycated haemoglobin, serum creatinine, homocysteine, total cholesterol, high-density lipoprotein cholesterol, triglycerides, smoking habits, and the use of medication. LDL cholesterol was calculated by the Friedewald formula,[21] except when the triglyceride level was > 4.55 mmol/L (n=23). Hypertension was defined as a blood pressure ≥ 140 mmHg systolic and/or ≥ 90 mmHg diastolic and/or the current use of antihypertensive medication. Subjects were classified as current cigarette smokers or non-smokers. The glomerular filtration rate was calculated according to Levey et al.[22]

Follow-up

For each subject, we determined whether or not death had occurred during follow-up, and if so, the date at which death occurred. Data on the subjects' vital status on 1 January 2003 were collected from the mortality register of the municipality of Hoorn. Of 51 subjects who had moved out of town, information on vital status was obtained from the new local municipalities. For all subjects who had died, the cause of death was

extracted from the medical records of the general practitioner and the hospital of Hoorn, and classified according to the 9th edition of the International Classification of Diseases.[23] Cardiovascular mortality was defined as codes 378 and 390-459. Information on cause of death could not be obtained for 33 (19%) of the deceased subjects and 1 subject was lost to follow-up.

Statistical analyses

Because markers of endothelial dysfunction and inflammatory activity show marked intra-individual (day-to-day) variation and because we measured these markers only once, the associations (if any) of endothelial dysfunction and inflammatory activity with other variables will tend to be underestimated. As a result of this, statistical power will be diminished. To address this concern, we created mean standard deviation scores (z-scores) for markers of endothelial dysfunction and chronic low-grade inflammation, and used these in regression analyses as described below. For each subject, each variable was expressed as standard deviations of difference from the population mean, which was calculated using all available data on the separate markers (n=608 and 610 [of 631] for the endothelial dysfunction and inflammation z-scores, respectively). The z-scores were calculated as the mean of these standard deviation scores as follows: (1) endothelial dysfunction z-score = {vWf + sVCAM-1} / 2; (2) inflammation z-score = {CRP + sICAM-1} / 2.

To assess to what extent the associations of T2D and IGM on the one hand with endothelial dysfunction and low-grade inflammation on the other were independent of other risk factors which are known to be associated with deteriorated glucose tolerance, we performed linear regression analyses. Endothelial dysfunction or inflammation z-scores were entered as dependent variables and T2D and IGM as independent variables, with adjustment for potential confounders. Results are described as regression coefficients (ß) with 95% confidence intervals.

To assess associations of markers of endothelial dysfunction and low-grade inflammation with risks of cardiovascular and all-cause mortality, we performed Kaplan-Meier and Cox proportional hazards multiple regression analyses. Because of the stratification procedure, we first adjusted for age, sex and glucose tolerance status in all models, and, subsequently, for other potential confounders. Variables measured on a continuous scale were used as such in the regression models, except for levels of sVCAM-1, CRP, and systolic and diastolic blood pressure, because of their non-linear association with mortality. Therefore, a high level of sVCAM-1 was defined as in the upper tertile (>1485 ng/mL); data on CRP were log transformed before analysis; and systolic and diastolic blood pressure were defined as high (≥140 mmHg and ≥ 90 mmHg, respectively) or low. In spite of the non-linear association of sVCAM-1 (all-cause) and CRP (both all-cause and cardiovascular) with mortality, additional analysis showed that associations between the z-scores and mortality were nevertheless best described as linear. To evaluate a possible interaction between glucose tolerance status

and endothelial dysfunction or low-grade inflammation, Cox regression analyses were performed with glucose tolerance status, the endothelial dysfunction or inflammation z-score, their product term, age, and sex in the model. A significant hazard ratio for the product term was considered indicative for interaction of glucose tolerance status with either low-grade inflammation or endothelial dysfunction. Results are described as relative risks (hazard ratios) with 95% confidence intervals.

To assess to what extent associations of T2D and IGM with cardiovascular mortality could in fact be explained by T2D- and IGM-associated endothelial dysfunction and low-grade inflammation, we performed Cox regression analyses without and with adjustments for the endothelial dysfunction and inflammation z-scores, and without and with potential interaction terms. Percentages explained were calculated using the regression coefficients instead of hazard ratios, because of the logarithmic character of the hazard ratio. All models were fitted comparing T2D and IGM to NGM, and T2D to IGM separately.

Two-sided P-values less than 0.05 were considered statistically significant, except for the interaction analyses, where p-values less than 0.10 were used.

Results

The median duration of follow-up was 11.7 years (range 0.5 – 13.2 years). After follow-up, 174 (55 NGM, 43 IGM, 76 T2D) of the 631 subjects had died, of whom 66 (38%; 17 NGM, 16 IGM, 33 T2D) had died of cardiovascular disease. Table 1 shows the baseline characteristics of the study population according to glucose tolerance status.

Glucose tolerance status is associated with endothelial dysfunction and low-grade inflammation

Table 2 and Figure I (appendix) show that T2D was significantly associated with both endothelial dysfunction and low-grade inflammation, whereas IGM was associated only with low-grade inflammation.

Higher levels of markers of endothelial dysfunction and low-grade inflammation are associated with greater mortality risks

Figure 1 and Table 3 show that, in general, higher levels of markers of endothelial dysfunction, low-grade inflammation, and their z-scores were associated with greater mortality risks. For example, the hazard ratio of cardiovascular and all-cause mortality associated with the inflammation z-score were 1.43 (1.17 to 1.77) and 1.27 (1.10 to 1.47) per SD difference. For endothelial dysfunction, the associations with cardiovascular and all-cause mortality were stronger in diabetic than in non-diabetic individuals (P-interaction = 0.064 and 0.028; Table 3). For example, the cardiovascular mortality hazard ratio associated with the endothelial dysfunction z-score was 1.87 (1.43 to 2.45) for diabetic individuals as compared to 1.23 (0.86 to 1.75) in non-diabetic individuals. For all-cause mortality, the hazard ratio was 1.41 (1.16 to 1.72) in diabetic

Table 1. Baseline characteristics of the study population according to glucose tolerance status

	NGM	IGM	T2D	P (trend)
No. (M/F)	258 (126/132)	179 (91/88)	194 (87/107)	
Conventional risk factors				
Age (year)	63 ± 7	64 ± 7	66 ± 7	0.001
HbA_{1c} (% of haemoglobin)	5.3 ± 0.5	5.6 ± 0.5	7.1 ± 1.8	<0.001
Hypertension (%)	25	43	55	<0.001
Diastolic blood pressure (mmHg)	81 ± 10	84 ± 10	83 ± 10	0.05
Systolic blood pressure (mmHg)	133 ± 18	142 ± 20	144 ± 19	<0.001
Current smokers (%)	30	22	24	0.15
Body mass index (kg/m^2)	25.9 ± 3.3	27.6 ± 3.7	28.7 ± 4.4	<0.001
HDL-cholesterol (mmol/L)	1.4 ± 0.4	1.3 ± 0.4	1.1 ± 0.3	<0.001
LDL-cholesterol (mmol/L)	4.6 ±1.0	4.6 ± 1.0	4.3 ± 1.1	0.003
Triglycerides (mmol/L)	1.3 (1.0 to 1.8)	1.6 (1.2 to 2.3)	2.0 (1.3 to 2.8)	<0.001
Homocysteine (umol/L)	11.2 (9.2 to 14.3)	12.2 (9.7 to 14.9)	11.1 (9.0 to 13.5)	0.47
Glomerular filtration rate (ml/min) ⁎	68.0 ± 11.3	67.9 ± 11.1	67.4 ± 13.8	0.60
Prior cardiovascular disease (%)	17	23	31	0.001
Markers of endothelial dysfunction and chronic low-grade inflammation				
vWf (%pooled plasma)	106 (90 to 131)	115 (93 to 150)	148 (108 to 176)	<0.001
sVCAM-1 (ng/mL)	1316 ± 377	1363 ± 420	1497 ± 540	<0.001
Endothelial dysfunction z-score (SD)	-0.23 ± 0.85	-0.05 ± 0.93	0.34 ± 1.15	<0.001†
CRP (mg/L)	1.30 (0.84 to 1.98)	2.11 (1.03 to 3.05)	2.43 (1.63 to 356)	<0.001
sICAM-1 (ng/mL)	448 ± 123	489 ± 166	520 ± 195	<0.001
Inflammation z-score (SD)	-0.29 ± 0.87	0.07 ± 1.01	0.32 ± 1.05	<0.001‡

Data are mean ± SD or median (interquartile range). Mean standard deviation scores (z-scores) for markers of endothelial dysfunction and chronic low-grade inflammation were created. For each subject, each variable was expressed as standard deviations of difference from the population mean, which was calculated using all available data on the separate markers (n=608 and 610 [of 631] for the endothelial dysfunction and inflammation z-scores, respectively). The z-scores were calculated as the mean of these standard deviation scores as follows: (1) endothelial dysfunction z-score = {vWf + sVCAM-1}/2; (2) inflammation z-score = {CRP + sICAM-1}/2.

⁎ According to the MDRD formula

† IGM vs. NGM P=0.050; T2D vs. IGM P<0.0001; T2D vs. NGM P<0.0001

‡ IGM vs. NGM P<0.0001; T2D vs. IGM P=0.021; T2D vs. NGM P<0.0001

NGM: normal glucose metabolism; IGM: impaired glucose metabolism; T2D: type 2 diabetes; M: male; F: female; HbA_{1c} : Haemoglobin A_{1c}; sVCAM-1: soluble vascular adhesion molecule-1; sICAM-1: soluble intercellular adhesion molecule-1; SD: standard deviation; vWf: von Willebrand factor; CRP: C-reactive protein

individuals and 1.09 (0.87 to 1.36) in non-diabetic individuals. Results were similar when individual markers were used instead of the endothelial dysfunction and inflammation z-scores (data not shown). Table I (appendix) shows that adjustment for potential confounders (hypertension, smoking, LDL cholesterol, HDL cholesterol, triglycerides, prior cardiovascular disease, body mass index, homocysteine, and glomerular filtration rate) did not markedly change the associations of the endothelial dysfunction and inflammation z-scores with cardiovascular and all-cause mortality.

Table 2. Associations between glucose tolerance status and the endothelial dysfunction and inflammation z-scores

Added variables		Regression coefficient (ß)			
		Endothelial dysfunction z-score		Inflammation z-score	
Model		T2D vs. NGM	IGM vs. NGM	T2D vs. NGM	IGM vs. NGM
1	Age and sex	0.521 ✱ † (0.337 to 0.705)	0.153 (-0.034 to 0.341)	0.594 ✱ ‡ (0.409 to 0.779)	0.357 ✱ (0.168 to 0.545)
2	Age, sex, hypertension, smoking, LDL cholesterol, and prior cardiovascular disease	0.395 ✱ † (0.204 to 0.585)	0.098 (-0.091 to 0.286)	0.490 ✱ (0.301 to 0.678)	0.313 § (0.127 to 0.500)
3	Model 2 and body mass index	0.350 ✱ ‡ (0.155 to 0.546)	0.080 (-0.110 to 0.269)	0.390 ✱ (0.198 to 0.583)	0.265 § (0.079 to 0.451)
4	Model 2 and HDL cholesterol	0.360 ✱ ‡ (0.164 to 0.556)	0.080 (-0.080 to 0.270)	0.392 ✱ (0.200 to 0.584)	0.267 § (0.082 to 0.452)
5	Model 2 and triglycerides	0.391 ✱ ‡ (0.191 to 0.591)	0.100 (-0.090 to 0.291)	0.384 ✱ (0.188 to 0.579)	0.269 § (0.083 to 0.455)
6	Model 2 and homocysteine	0.414 ✱ ‡ (0.225 to 0.603)	0.083 (-0.104 to 0.269)	0.496 ✱ (0.307 to 0.685)	0.309 § (0.122 to 0.495)
7	Model 2 and glomerular filtration rate ‖	0.431 ✱ ‡ (0.243 to 0.618)	0.097 (-0.090 to 0.283)	0.486 ✱ (0.294 to 0.677)	0.325 § (0.134 to 0.515)

Regression coefficients ß and 95% confidence intervals were obtained by linear regression analysis with endothelial dysfunction or inflammation z-scores as dependent variables and IGM or T2D as independent variables. Model 1: adjusted for stratification variables; model 2: adjusted for stratification variables and hypertension, smoking, LDL cholesterol, and prior cardiovascular disease; models 3-6, plus adjusted for other potential confounders. The same analysis was also performed with NGM and T2D as independent variables to compare T2D with IGM.

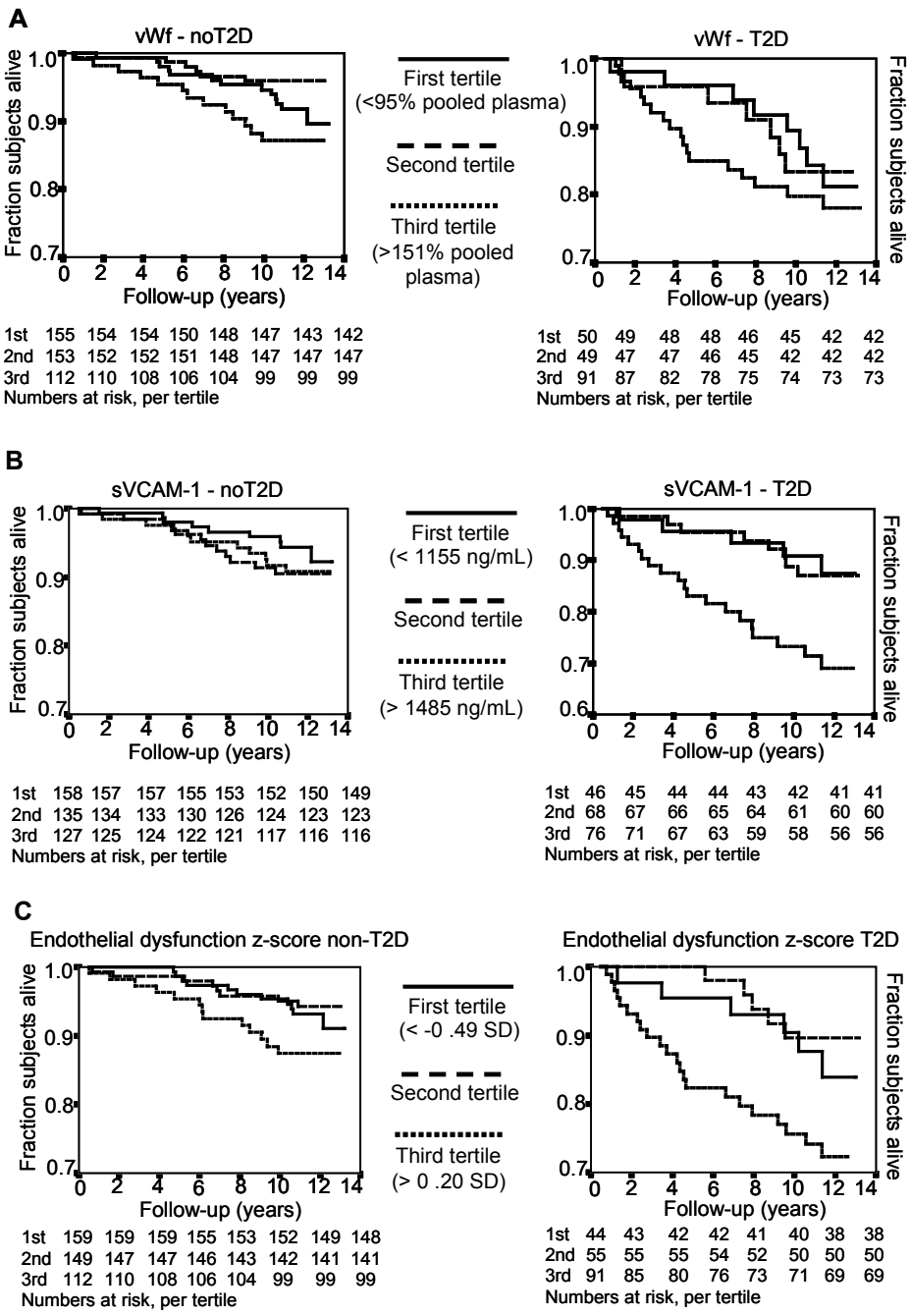
✱ P < 0.0001 vs. NGM; † P < 0.0001 vs. IGM

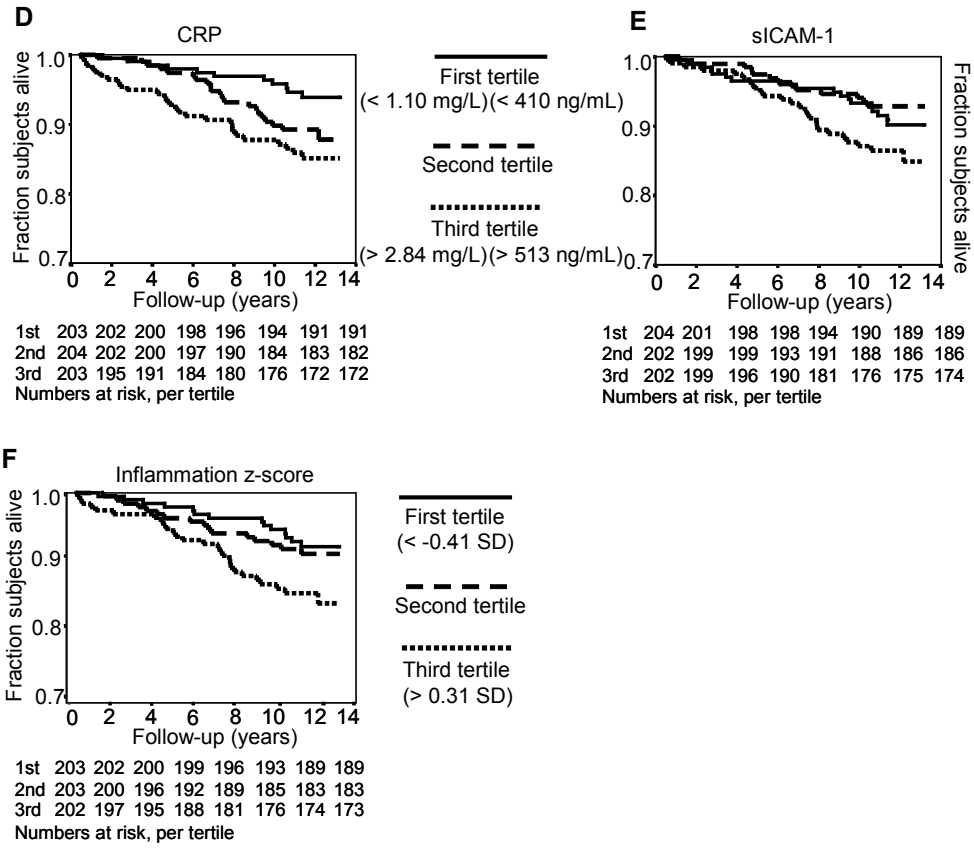
‡ P < 0.025 vs. IGM; § P < 0.025 vs. NGM; other P-values > 0.1

‖ According to the MDRD formula

NGM: normal glucose metabolism; IGM: impaired glucose metabolism; T2D: type 2 diabetes

Figure 1. Cardiovascular survival (Kaplan-Meier method) according to markers of endothelial dysfunction and chronic low-grade inflammation





Endothelial dysfunction and low-grade inflammation explain much of the cardiovascular mortality risks associated with T2D

Table II (appendix) shows that T2D was significantly associated with both cardiovascular and all-cause mortality (2.74 [1.52 to 4.92] and 1.90 [1.34 to 2.69], respectively), but IGM was not (1.25 [0.63 to 2.48] and 1.05 [0.70 to 1.56], respectively). Adjustment for the endothelial dysfunction and low-grade inflammation z-scores reduced the magnitude of the association between T2D and cardiovascular mortality by 34% and 25%, respectively. Together, the z- scores explained 43% of the cardiovascular mortality risk associated with T2D (Table II, Figure 2). Results were similar when adjusted for the individual markers instead of the endothelial dysfunction and inflammation z-scores (data not shown). Adjustment for traditional risk factors as hypertension, smoking, LDL cholesterol, body mass index, and prior cardiovascular disease did not reduce the magnitude of the association between T2D and cardiovascular mortality to this extent, please see Table II (appendix).

Table 3. Hazard ratios of cardiovascular and all-cause mortality associated with markers of endothelial dysfunction and chronic, low-grade inflammation, and with potential confounders

	Contrast for which hazard ratio is presented		Cardiovascular mortality hazard ratio	All-cause mortality hazard ratio
Markers of endothelial dysfunction and chronic low-grade inflammation				
VWf (%pooled plasma)	Per SD increase *	T2D	1.31 (1.00 to 1.72)	1.10 (0.91 to 1.34)
		No T2D	1.23 (0.88 to 1.73)	1.26 (1.04 to 1.54)
sVCAM-1 (ng/mL)	High vs. low †	T2D	2.87 (1.42 to 5.80)	2.16 (1.37 to 3.41)
		No T2D	1.05 (0.51 to 2.17)	0.72 (0.46 to 1.14)
Endothelial dysfunction z-score (SD)	Per SD increase *	T2D	1.87 (1.43 to 2.45)	1.41 (1.16 to 1.72)
		No T2D	1.23 (0.86 to 1.75)	1.09 (0.87 to 1.36)
CRP (mg/L)	Doubling ‡		1.63 (1.04 to 2.57)	1.27 (0.97 to 1.67)
sICAM-1 (ng/mL)	Per SD increase *		1.28 (1.09 to 1.50)	1.22 (1.08 to 1.37)
Inflammation z-score (SD)	Per SD increase *		1.43 (1.17 to 1.77)	1.27 (1.10 to 1.47)
Potential confounders				
Male sex (%)	Yes vs. no		1.46 (0.90 to 2.38)	1.62 (1.20 to 2.19)
Age (year)	Per SD increase *		2.22 (1.65 to 2.98)	1.93 (1.61 to 2.29)
T2D (%)	T2D vs. NGM		2.74 (1.52 to 4.92)	1.90 (1.34 to 2.69)
IGM (%)	IGM vs. NGM		1.25 (0.63 to 2.48)	1.05 (0.70 to 1.56)
HbA_{1c} (% of haemoglobin)	Per SD increase *		1.12 (0.89 to 1.42)	1.17 (1.00 to 1.36)
Hypertension (%)	Yes vs. no		1.97 (1.17 to 3.29)	1.58 (1.16 to 2.15)
Diastolic blood pressure	High vs. low †		1.08 (0.61 to 1.91)	1.10 (0.78 to 1.57)
Systolic blood pressure	High vs. low †		1.66 (1.00 to 2.75)	1.32 (0.96 to 1.82)
Current smokers (%)	Yes vs. no		1.62 (0.93 to 2.83)	1.65 (1.18 to 2.30)
Body mass index (kg/m^2)	Per SD increase *		1.11 (0.87 to 1.42)	1.13 (0.97 to 1.32)
HDL-cholesterol (mmol/L)	Per SD decrease *		1.18 (0.85 to 1.65)	1.05 (0.88 to 1.25)
LDL-cholesterol (mmol/L)	Per SD increase *		1.12 (1.08 to 1.17)	1.02 (0.88 to 1.18)
Triglycerides (mmol/L)	Per SD increase *		1.16 (0.91 to 1.48)	1.18 (1.05 to 1.32)
Homocysteine (umol/L)	Per SD increase *		1.09 (0.93 to 1.29)	1.05 (0.92 to 1.19)
Glomerular filtration rate	Per SD decrease *		1.58 (1.22 to 2.05)	1.32 (1.12 to 1.57)
Prior cardiovascular	Yes vs. no		2.25 (1.37 to 3.70)	1.74 (1.26 to 2.38)

Hazard ratios and 95% confidence intervals were obtained by Cox regression analysis after adjustment for age, sex, and glucose tolerance status, except when analyses were stratified for glucose tolerance status because of interaction between glucose tolerance status and endothelial dysfunction, or when glucose tolerance status was the variable under consideration.

* SDs for von Willebrand factor, 67.6%; for endothelial dysfunction z-score, 1; for sICAM-1, 162.9 ng/mL; for inflammation z-score, 1; for age, 7.2 years; for HbA_{1c} , 1.3%; for body mass index , 4.0 kg/m^2; for HDL-cholesterol, 0.4 mmol/L; for LDL-cholesterol, 1.1 mmol/L; for triglycerides, 1.3 mmol/L; for homocysteine, 5.8 umol/L; and for glomerular infiltration rate, 12.1 ml/min.

† sVCAM-1 high ≥1485 ng/mL, low < 1484 ng/mL; diastolic blood pressure high ≥90 mmHg, low < 90 mmHg; systolic blood pressure high ≥140 mmHg, low < 140 mmHg

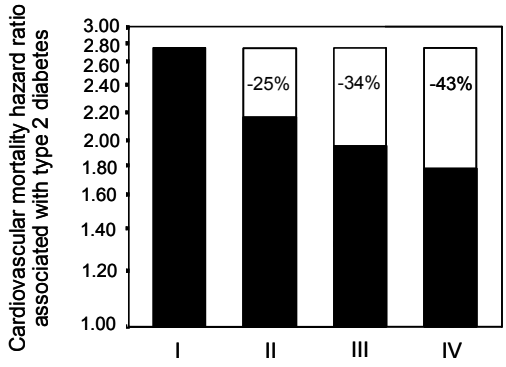
‡ Data on CRP were log transformed before analysis, because of its nonlinear association with mortality

§ According to the MDRD formula

Additional analyses

Exclusion of individuals with impaired fasting glucose (n=29) did not affect the results (data not shown). The following additional adjustments also did not materially affect our results: analyses using waist or waist-to-hip ratio instead of BMI; analyses using the creatinine clearance according to the Cockcroft-Gault criteria instead of the glomerular filtration rate; and analyses that adjusted for microalbuminuria. Hazard ratios remained constant over time (data not shown).

Figure 2. Hazard ratio of cardiovascular mortality associated with type 2 diabetes after adjustment for stratification variables (I), low-grade inflammation (II), endothelial dysfunction (III), and both low-grade inflammation and endothelial dysfunction (IV)



Discussion

Our study on endothelial dysfunction and low-grade inflammation in individuals without and with T2D had three main findings. Firstly, T2D was associated with both endothelial dysfunction and low-grade inflammation, whereas IGM was associated only with low-grade inflammation. These findings were independent of other risk factors that accompany T2D or IGM. Secondly, endothelial dysfunction and low-grade inflammation were associated with greater risks of cardiovascular mortality, especially in T2D. Thirdly, T2D-associated endothelial dysfunction and low-grade inflammation explained about 43% of the greater cardiovascular mortality risk conferred by T2D.

Strengths of our study include its population-based design; the long follow-up (up to 13 years); the limited loss to follow-up; and the extensive characterization of participants at baseline. In addition, the results were robust and consistent across the markers of endothelial dysfunction and inflammation used.

As expected, T2D was associated with both endothelial dysfunction and low-grade inflammation.[12,18,24] In contrast, IGM was associated only with low-grade

inflammation, which is in agreement with previous studies that have shown a much clearer association of IGM with low-grade inflammation than with endothelial dysfunction,[25-29] which to some extent appears to depend on the endothelial function marker used.[28-36] Taken together, these data suggest that endothelial dysfunction is not universal in IGM and may depend on other factors not identified in these studies.

Endothelial dysfunction and low-grade inflammation were associated with higher risks of cardiovascular mortality, consistent with previous studies.[7,18-20,37] Importantly, we show that, for endothelial dysfunction, these associations were stronger in diabetic than in non-diabetic individuals; were independent of other cardiovascular risk factors; remained present during up to 13 years of follow-up; and appeared mutually independent,[7,37] indicating that they may represent largely distinct pathways of disease and therefore distinct targets for intervention.

Both endothelial dysfunction and low-grade inflammation appeared to explain parts of the increased mortality risks associated with T2D. However, the role of endothelial dysfunction seems especially relevant because of its interaction with T2D. Taken together, our data suggest that treatments to improve the cardiovascular prognosis of individuals with T2D should focus on improving endothelial function and decreasing chronic inflammation. The causes of endothelial dysfunction and low-grade inflammation in T2D remain incompletely understood and may include not only obesity, hypertension, dyslipidaemia, insulin resistance, and hyperglycaemia (the metabolic syndrome), but also advanced glycation endproducts.[12] In addition, endothelial dysfunction and low-grade inflammation may precede and contribute to the occurrence of T2D.[38]

In the present study, IGM was not clearly associated with an increased mortality risk (although the confidence limits show that we could not exclude any such associations with great certainty), and we therefore could not test the influence of endothelial dysfunction or low-grade inflammation. Other reports from the Hoorn Study have shown that 2-hour post-load plasma glucose concentrations do predict cardiovascular and all-cause mortality, but mostly in the diabetic range.[2,39] Other studies on IGM and risk of mortality have reported inconsistent results.[40-46]

Our study has several limitations. Firstly, its relatively small size and consequently limited power may have obscured more subtle associations. Secondly, the incomplete assessment of endothelial function and inflammatory activity may have increased non-differential misclassification, leading to an underestimation of the hazard ratios presented here. However, our results were robust and consistent with previous experience. Thirdly, we used sICAM-1 as a marker of inflammation, even though sICAM-1 can be regarded as a marker of both endothelial function and inflammation.[16,47] However, to classify sICAM-1 as a marker of inflammation can be considered the most conservative alternative, as sICAM-1 is not selectively derived from endothelial cells, but originates from leukocytes as well. sICAM-1 upregulation, however, is driven by inflammatory cytokines, such as TNF-α and IL-8, resulting in the activation of nuclear factor kappa B.[48] Importantly, additional analyses showed that our conclusions remain unchanged when sICAM-1 is classified as a marker of endothelial function (data not shown). Fourthly, we studied Caucasian individuals and the results

therefore are not necessarily valid for other ethnicities. Fifthly, an assumption in the construction of the z-scores is that its components are equally important, which is not necessarily true. Nevertheless, z-scores have the considerable merit of increased precision, as demonstrated by the smaller confidence intervals of the z-scores as compared to those of the individual markers, and, possibly, of increased validity, as these z-scores address various aspects of endothelial dysfunction and inflammation, respectively. Finally, because traditional risk factors were measured only once, we may to some extent have underestimated their associations with mortality, although previous analyses from the Hoorn Study have shown that traditional risk factors, even if measured only once, do in fact predict mortality.[49]

In conclusion, we have shown that T2D is associated with both endothelial dysfunction and low-grade inflammation, whereas IGM is associated only with low-grade inflammation; that endothelial dysfunction and low-grade inflammation are associated with greater risks of cardiovascular mortality, especially in T2D; and that T2D-associated endothelial dysfunction and low-grade inflammation can explain about 43% of the higher cardiovascular mortality risk conferred by T2D. These data emphasize the necessity of randomized controlled trials of strategies that aim to decrease cardiovascular disease risk by improving endothelial function and decreasing low-grade inflammation – especially in T2D, in whom endothelial dysfunction is particularly ominous and in whom both endothelial dysfunction and low-grade inflammation are highly prevalent.

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L.M.B., J.M.D., R.J.H., G.N. and C.D.A.S. are responsible for the design and management of the Hoorn Study. J.d.J., P.J.K. and J.M.D. did the statistical analyses. JdJ and CS drafted the manuscript. All authors contributed to the final version of the manuscript. All authors have seen and approved the final version.

The corresponding author had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of Interests

No conflict of interests exists.

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Appendix

Table I. Hazard ratios of cardiovascular and all-cause mortality associated with the endothelial dysfunction z-score and the inflammation z-score: multivariate adjustments

	Added variables	Cardiovascular mortality hazard ratio (95% CI)		
		Endothelial dysfunction z-score		Inflammation z-score
		T2D	No T2D	All subjects
1	Stratification variables	1.87 (1.43-2.45)	1.23 (0.86-1.75)	1.43 (1.17-1.77)
2	1 and hypertension, smoking, LDL cholesterol, prior cardiovascular disease	1.84 (1.42-2.40)	1.11 (0.76-1.62)	1.39 (1.06-1.74)
3	2 and body mass index	1.80 (1.37-2.34)	1.19 (0.82-1.73)	1.35 (1.06-1.71)
4	2 and HDL cholesterol	1.85 (1.42-2.41)	1.18 (0.82-1.71)	1.37 (1.09-1.72)
5	2 and triglycerides	1.85 (1.42-2.41)	1.18 (0.82-1.70)	1.40 (1.11-1.75)
6	2 and homocysteine	1.84 (1.42-2.40)	1.16 (0.79-1.70)	1.39 (1.10-1.74)
7	2 and glomerular filtration rate *	1.80 (1.37-2.37)	1.08 (0.73-1.60)	1.42 (1.12-1.81)
8	2 and inflammation or endothelial dysfunction z-score	1.66 (1.19-2.31)	1.11 (0.76-1.62)	1.23 (0.97-1.56)
	Added variables	All-cause mortality hazard ratio (95% CI)		
		Endothelial dysfunction z-score		Inflammation z-score
		T2D	No T2D	All subjects
1	Stratification variables	1.41 (1.16-1.72)	1.09 (0.87-1.36)	1.27 (1.10-1.47)
2	1 and hypertension, smoking, LDL cholesterol, prior cardiovascular disease	1.41 (1.16-1.72)	1.06 (0.85-1.34)	1.20 (1.03-1.40)
3	2 and body mass index	1.39 (1.14-1.69)	1.05 (0.84-1.33)	1.17 (1.00-1.37)
4	2 and HDL cholesterol	1.41 (1.16-1.72)	1.06 (0.85-1.33)	1.19 (1.02-1.39)
5	2 and triglycerides	1.40 (1.14-1.71)	1.07 (0.86-1.33)	1.18 (1.00-1.38)
6	2 and homocysteine	1.41 (1.16-1.72)	1.06 (0.84-1.33)	1.20 (1.02-1.39)
7	2 and glomerular filtration rate *	1.39 (1.14-1.70)	0.98 (0.77-1.25)	1.20 (1.02-1.40)
8	2 and inflammation or endothelial dysfunction z-score	1.29 (1.02- 1.63)	1.03 (0.82-1.30)	1.13 (0.97-1.33)

Hazard ratios and 95% confidence intervals were obtained by Cox regression analysis. Contrasts are per SD increase in endothelial dysfunction score/inflammation z-score. Model 1: adjusted for stratification variables: age, sex, and glucose tolerance status; model 2: adjusted for stratification variables, plus hypertension, smoking, LDL cholesterol, and prior cardiovascular disease; models 3-7: as model 3, plus adjusted for other potential confounders; model 8: adjusted for stratification variables, plus hypertension, smoking, LDL cholesterol and prior cardiovascular disease, and either inflammation or endothelial dysfunction z-scores.

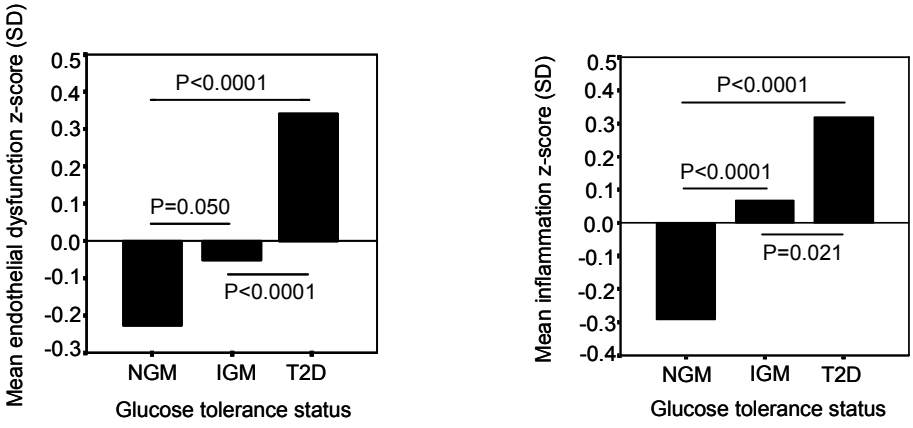
* According to the MDRD formula; CI: confidence interval; T2D: type 2 diabetes; SD: standard deviation

Table II. Hazard ratios of cardiovascular and all-cause mortality associated with type 2 diabetes and IGM: effect of adjustment for the inflammation and endothelial dysfunction z-scores, potential confounders, and both potential confounders and z-scores

	Added variables	Cardiovascular mortality hazard ratio (95% CI)	
		T2D vs. NGM #	IGM vs. NGM
1	Age, sex	2.74 (1.52-4.92) ∗	1.25 (0.63-2.48)
2	1 and inflammation z-score	2.13 (1.14-3.99) ∗	1.12 (0.55-2.26)
3	1 and endothelial dysfunction z-score, and interaction term †	1.95 (1.01-3.73)	1.28 (0.63-2.57)
4	3 and inflammation z-score	1.78 (0.91-3.45) #	1.16 (0.57-2.34)
5	1 and hypertension, smoking, LDL cholesterol, and prior cardiovascular disease	2.14 (1.16-3.96) ∗	1.05 (0.52-2.11)
6	5 and body mass index	2.03 (1.07-3.84) ∗	1.03 (0.51-2.11)
7	5 and HDL cholesterol	1.90 (1.00-3.61) ∗	0.97 (0.47-1.99)
8	5 and triglycerides	2.00 (1.05-3.82) ∗	1.01 (0.50-2.06)
9	5 and homocysteine	2.14 (1.16-3.96) ∗	1.04 (0.52-2.11)
10	5 and glomerular filtration rate §	2.15 (1.16-3.98) ∗	1.05 (0.52-2.13)
11	4 and 5	1.47 (0.74-2.93) #	0.98 (0.47-2.03)
	Added variables	All-cause mortality hazard ratio (95% CI)	
		T2D vs. NGM «	IGM vs. NGM
1	Age, sex	1.90 (1.34-2.69) ‡	1.05 (0.70-1.56)
2	1 and inflammation z-score	1.60 (1.11-2.31) ‡	0.90 (0.59-1.36)
3	1 and endothelial dysfunction z-score, and interaction term †	1.60 (1.10-2.32) ∗	0.98 (0.65-1.47)
4	3 and inflammation z-score	1.49 (1.02-2.17) ∗	0.91 (0.60-1.38)
5	1 and hypertension, smoking, LDL cholesterol, and prior cardiovascular disease	1.57 (1.09-2.27) ∗	0.96 (0.64-1.45)
6	5 and body mass index	1.46 (1.00-2.15) ∗	0.93 (0.62-1.41)
7	5 and HDL cholesterol	1.52 (1.04-2.24) ∗	0.95 (0.63-1.43)
8	5 and triglycerides	1.34 (0.91-1.99) ∗	0.90 (0.60-1.36)
9	5 and homocysteine	1.57 (1.09-2.27) ∗	0.96 (0.64-1.44)
10	5 and glomerular filtration rate §	1.62 (1.11-2.36) ∗	0.97 (0.64-1.47)
11	4 and 5	1.32 (0.89-1.95) ∗	0.86 (0.36-1.32)

Hazard ratios and 95% confidence intervals were obtained by Cox regression analysis. Model 1: adjusted for stratification variables; model 2: as model 1, plus adjusted for the inflammation z-score; model 3: as model 1, plus adjusted for the endothelial dysfunction z-score and interaction term (endothelial dysfunction z-score x diabetes presence); model 4: as model 3, plus adjusted for the inflammation z-score; model 5: as model 1, plus adjusted for hypertension, smoking, LDL cholesterol, and prior cardiovascular disease; models 6-10: as model 5 plus adjusted for other potential confounders; model 11: adjusted for stratification variables, inflammation z-score, endothelial dysfunction z-score and interaction term (endothelial dysfunction z-score x diabetes presence), and hypertension, smoking, LDL cholesterol, and prior cardiovascular disease. The percentages of the cardiovascular mortality risk explained by endothelial dysfunction and low-grade inflammation were calculated using the regression coefficients instead of the hazard ratios. For example, after adjustment for the inflammation z-score the hazard ratio changed from 2.74 (regression coefficient Ln(2.74)=1.007) to 2.13 (regression coefficient Ln(2.13)=0.756), representing a 25% decrease. ∗P<0.05 vs. IGM; ‡ P<0.005 vs. IGM; other P values >0.05; † Interaction term: endothelial dysfunction z-score x diabetes presence (interaction term regression coefficient 0.41 and 0.27 for cardiovascular and all-cause mortality, respectively); § According to the MDRD formula; CI: confidence interval; T2D: type 2 diabetes; IGM: impaired glucose metabolism; NGM: normal glucose metabolism

Figure I. Mean endothelial dysfunction and inflammation z-scores according to glucose tolerance status



NGM normal glucose metabolism; IGM impaired glucose metabolism; T2D type 2 diabetes

Chapter 3

Effects of short-term treatment with metformin on markers of endothelial function and inflammatory activity in type 2 diabetes mellitus: a randomised, placebo-controlled trial

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Abstract

Objectives

The U.K. Prospective Diabetes Study showed that treatment with metformin decreases macrovascular morbidity and mortality independent of glycaemic control. We hypothesised that metformin may achieve this by improving endothelial function and chronic, low-grade inflammation.

Design

The HOME trial is a double-blind trial, in which all patients were randomised to receive either metformin or placebo in addition to insulin therapy. At the beginning and the end of a 16-week treatment period fasting blood samples were drawn and a physical examination was carried out.

Setting

The trial was conducted in the outpatient clinics of three non-academic hospitals (Hoogeveen, Meppel, and Coevorden; the Netherlands).

Subjects

Patients were included if they were between 30 and 80 years of age and received a diagnosis of diabetes after the age of 25, had never had an episode of ketoacidosis, and their blood-glucose-lowering treatment previously consisted of oral agents but now only consisted of either insulin (n=345) or insulin and metformin (n=45). We excluded pregnant women and women trying to become pregnant, patients with a Cockroft-Gault-estimated creatinine clearance < 50 ml/min, or low plasma cholinesterase (reference value < 3.5 units/l), patients with congestive heart failure (New York Heart Association class III/IV), or patients with other serious medical or psychiatric disease. 745 eligible patients were approached, 390 gave informed consent and were randomised (196 metformin, 194 placebo). 353 patients completed 16 weeks of treatment (171 metformin, 182 placebo).

Main outcome measures

The HOME trial was designed to study the metabolic and cardiovascular effects of metformin during a follow-up of 4 years. Presented here are the results of an interim analysis after 16 weeks of treatment.

Results

As compared to placebo, metformin treatment was associated with an increase in urinary albumin excretion of 21% ([-1 to +48]; p=0.06); a decrease in von Willebrand factor of 6% ([-10 to -2]; p=0.0007); a decrease in soluble vascular cell adhesion molecule-1 of 4% ([-7 to -2]; p=0.0002); a decrease in soluble E-selectin of 6% ([-10 to -2]; p=0.008); a decrease in tissue-type plasminogen activator of 16% ([-20 to -12]; p<0.0001); and a decrease in plasminogen activator inhibitor-1 of 20% ([-27 to -10];

p=0.0001). These changes could not be explained by metformin-associated changes in glycaemic control, body weight, or insulin dose. Markers of inflammation, i.e. C-reactive protein and soluble intercellular adhesion molecule-1, did not change with metformin treatment.

Conclusions

In patients with type 2 diabetes treated with insulin, metformin treatment was associated with improvement of endothelial function, which was largely unrelated to changes in glycaemic control, but not with chronic, low-grade inflammation.

Introduction

In type 2 diabetes mellitus, treatment with metformin has been associated with a decrease in macrovascular morbidity and mortality, which appears to be independent of the improvement in glycaemic control, as was demonstrated in the U.K. Prospective Diabetes Study (UKPDS).[1] This observation suggests that metformin may affect the risk of atherothrombotic disease through mechanisms other than the lowering of blood glucose.

Two key features in the pathophysiology of atherothrombosis are dysfunction of the vascular endothelium and chronic, low-grade inflammation of the vascular wall.[2] Indeed, observational studies have found strong associations between markers of endothelial dysfunction and chronic, low-grade inflammation on the one hand and increased risk of atherothrombotic disease on the other.[3, 4]

These data raise the question of whether metformin can improve endothelial function and decrease inflammatory activity, and thereby decrease risk of atherothrombotic disease. There is some evidence from controlled studies that this may be the case,[5-8] but most studies focused on markers of fibrinolysis only,[5, 6, 8] which may or may not reflect endothelial function.[9] The randomised, placebo-controlled trial "Hyperinsulinaemia: the Outcome of its Metabolic Effects" (HOME) was designed to investigate whether metformin, as compared to placebo but at a similar level of glycaemic control, decreases cardiovascular morbidity in patients with type 2 diabetes treated with insulin during a planned follow-up of 4 years. In view of the above considerations, we studied the effects of metformin on markers of endothelial function and low-grade inflammation in an interim analysis after 16 weeks of treatment. In addition, we explored biochemical mechanisms that might mediate metformin-associated improvements in endothelial function and inflammatory activity (if any).

Material and methods

Patients and procedures

The HOME trial was designed to study the metabolic and cardiovascular effects of metformin in insulin-treated type 2 diabetic patients.[10] We aimed to include 400 patients with type 2 diabetes mellitus between 30 and 80 years of age who had received a diagnosis of diabetes after the age of 25, who had never had an episode of ketoacidosis, and whose blood-glucose-lowering treatment had previously consisted of oral agents but now only consisted of either insulin (n=345) or insulin and metformin (n=45). We excluded pregnant women and women trying to become pregnant, patients with a Cockcroft-Gault-estimated creatinine clearance < 50 ml/min,[11] or low plasma cholinesterase (reference value < 3.5 units/l)[12] as a marker of liver failure. Patients with congestive heart failure (New York Heart Association class III/IV) or other serious medical or psychiatric disease were excluded as well.

All patients gave written informed consent. The medical ethical committees of the three participating hospitals approved the trial protocol. The trial has been and is being conducted in accordance with the Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) dated 17 July 1996 and in accordance with the Declaration of Helsinki (Edinburgh, 2000).

Study design

The HOME trial was conducted in the outpatient clinics of three non-academic hospitals (Hoogeveen, Meppel, and Coevorden). All patients were treated with insulin only for 12 weeks; four times daily (Actrapid preceding the three meals and Insulatard ante noctem; Novo Nordisk, Alphen a/d Rijn, the Netherlands) or twice daily (Mixtures of Actrapid (10-50%) and Insulatard (90-50%) preceding breakfast and dinner; Novo Nordisk). After these 12 weeks, the 16-week short-term active treatment phase began at the start of which all subjects were randomly assigned to receive either metformin or placebo in addition to insulin therapy. All patients were numbered in order of study entry and were given trial medication with the same number. The boxes and tablets of metformin had a similar appearance. Each participant successively increased the dose from one to finally three tablets of 850 mg a day, if tolerated. The first tablet was taken at bedtime, the second at breakfast, and the third at dinner. The treatment goals were fasting plasma glucose levels between 4 and 7 mmol/l and postprandial glucose levels between 4 and 10 mmol/l. At the beginning and the end of this 16-week short-term active treatment phase, fasting blood samples were drawn, a physical examination was carried out, and a complete medical history was taken. We have previously reported results on glycaemic control, weight gain, and insulin dose,[10] vitamin B12, folate, and homocysteine,[13] and ambulatory blood pressure.[14]

Laboratory investigations

The laboratories of the three hospitals used standard analytical methods with the same reference values for laboratory measurements on fasting plasma glucose, haemoglobin A_{1c} (HbA_{1c}), fasting lipid concentrations, and urinary albumin excretion. Plasma glucose levels were determined using an automated glucose oxidase method (Hitachi 917; Roche, Basel) in Hoogeveen and Meppel. HbA_{1c} (normal value 4.0 – 6.0%) was measured by high-performance liquid chromatography in Hoogeveen and by an immunoturbidimetric method (Unimate; Roche) in Meppel. Fasting lipid concentrations were assessed by standard methods. The Coevorden hospital used dry chemistry for all above-mentioned laboratory measurements (Orthoclinical Diagnostics; Johnson and Johnson, Rochester, NY). Plasma LDL cholesterol was directly determined by the N-geneous TM assay (Genzyme, Cambridge, MA). Urinary albumin excretion (at baseline the mean of 3 fresh morning urine collections) was measured by immunoturbidimetry (Roche Diagnostics) in Meppel and Hoogeveen and by means of nephelometry (BN ProSpec, Dade Behring) in Coevorden. Urinary albumin excretion was expressed as the albumin-to-creatinine ratio. Method comparison according to Passing and Bablok[15, 16] showed no significant deviation between these methods. In addition, by means of a

randomised block test, no significant difference in HbA_{1c} values between the three hospitals was found.

We measured plasma von Willebrand factor (vWf) antigen and C-reactive protein (CRP) with highly sensitive in-house sandwich enzyme immunoassays. Rabbit anti-human vWf or CRP immunoglobulins were used as catching antibodies; peroxidase-conjugated rabbit anti-human vWf or CRP immunoglobulins were used as detecting antibodies (Dako, Copenhagen, Denmark). O-Phenylenediamine (Sigma Chemical Co., St. Louis, USA) acted as substrate for both plasma vWf and CRP antigen. Levels of vWf are expressed as percentage of antigen levels in normal pooled plasma, which is defined as 100%. The intra- and inter-assay coefficients of variation were 2.0% and 5.7% for plasma vWf antigen and 3.9% and 8.7% for CRP, respectively.

We measured plasma levels of soluble (s) vascular cell adhesion molecule-1 (VCAM-1; Diaclone, Besançon, France), soluble intercellular adhesion molecule-1 (ICAM-1; Diaclone, Besançon, France), soluble E-selectin (Bender MedSystems, Vienna, Austria), tissue-type plasminogen activator (t-PA) antigen (Biopool International, Umeå, Sweden), and plasminogen activator inhibitor-1 (PAI-1) antigen (Innogenetics, Gent, Belgium) in duplicate by use of commercially available ELISA kits. The intra- and inter-assay coefficients of variation were 4.4% and 4.6% for sVCAM-1; 4.0% and 7.4% for sICAM-1; 3.1% and 11.9% for sE-selectin; 2.8% and 7.5% for tPA; and 2.8% and 8.2% for PAI-1, respectively.

LDL particle size was measured by high-performance gel-filtration chromatography (HPGC) with fluorescence detection after postcolumn labelling with parinaric acid, a fluorescent lipid probe.[17, 18] Both intra- and inter-assay coefficients of variation were < 0.25%. Samples used for the determination of vWf, CRP, sVCAM-1, sICAM-1, t-PA, PAI-1, and LDL particle size were stored at -80 °C until subsequent analysis.

We considered urinary albumin excretion and plasma levels of vWf, sVCAM-1, sE-selectin, t-PA and PAI-1 as markers of endothelial function[19-21] and plasma CRP and sICAM-1 as markers of inflammatory activity.[21-23]

Statistical analyses

We included only subjects with complete data sets (n=313). All data with a skewed distribution were log-transformed before analysis.

The endpoint of interest was the percentage change of each variable from baseline, and the differences in these changes between the metformin and the placebo group. The differences between the metformin and placebo group were tested by means of a Student's T-test on log-transformed values. As log values are not directly interpretable, the antilogs are reported instead. In case of log-transformed values, data are given as geometric mean (95% CI).

We used multiple linear regression analysis to investigate whether metformin-associated improvements in markers of endothelial function and markers of

inflammation, if any, were independent of changes in HbA_{1c}, fasting or postprandial plasma glucose, insulin dose, BMI, and LDL cholesterol concentration or particle size. A P-value <0.05 was considered statistically significant.

Results

Patients

We screened the medical files of all three participating outpatient clinics and identified 745 eligible patients (Figure 1). They were all invited to enrol in the trial and 390 subjects gave written informed consent. The subjects were subsequently randomised to receive either metformin (196 subjects) or placebo (194 subjects). Of these 390 patients, 37 dropped out; 25 in the metformin group and 12 in the placebo group. Two patients never took their medication (placebo 1, metformin 1); 9 patients withdrew their consent (placebo 5, metformin 4), and 26 experienced adverse effects (placebo 6, metformin 20). Of these 26 patients, 11 experienced diarrhoea (placebo 2, metformin 9); 5 encountered flatulence (placebo 1, metformin 4); 4 suffered fatigue (placebo 1, metformin 3); 1 experienced pruritus (metformin); 1 had headaches (metformin); 1 had pyrosis (placebo); 1 had nausea (metformin), 1 had a myocardial infarction (placebo), and 1 patient died suddenly (metformin).

Figure 1. HOME trial flow chart

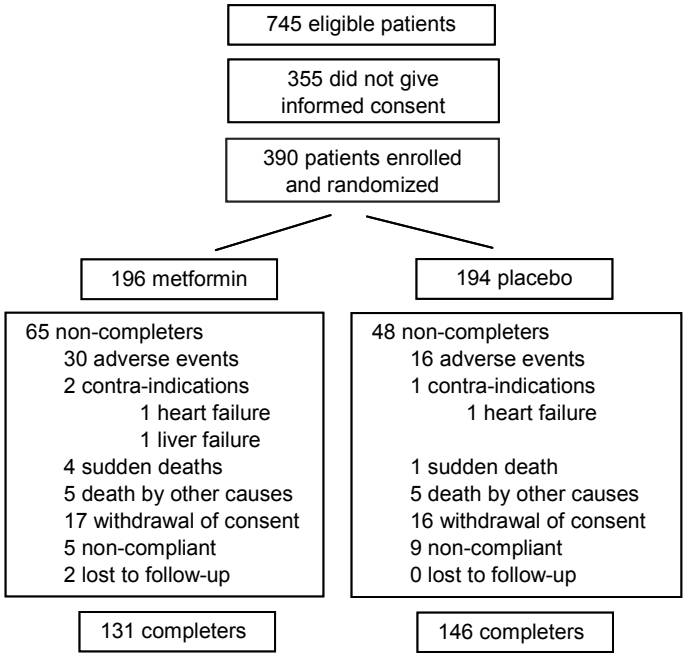

Table 1 shows the baseline characteristics at the start of the short-term active treatment phase. Patients randomised to receive metformin were slightly older than patients randomised to receive placebo (63.2 ± 9.8 vs. 58.9 ± 11.1 years), but other characteristics were comparable between the two groups. There were no large differences in the use of acetyl salicylic acid (ASA) or type of anti-hypertensive medication used (Table 1).

Table 1. Baseline characteristics

	Placebo (n=163)	Metformin (n=100)
Demography		
Men/women *n*	84/79	52/48
Age *(years)*	59 (11)	63 (9)
Currently smoking *n (%)*	50 (31)	31 (21)
Duration of diabetes *(years)*	12 (8)	14 (8)
Insulin treatment *(years)*	6 (6)	6 (7)
Diabetic complications		
Cardiovascular *n (%)*	53 (29)	59 (35)
Retinal coagulation and (or) cataract	25 (14)	35 (22)
Amputation *n (%)*	3 (2)	4 (2)
Paraesthesias *n (%)*	79 (43)	83 (49)
Concomitant medication		
Acetyl salicylic acid *n (%)*	25 (15)	29 (19)
Lipid-lowering drugs *n (%)*	36 (20)	34 (20)
Blood-pressure-lowering drugs *n (%)*	73 (40)	88 (51)
Enalapril	27 (17)	25 (17)
Losartan	3 (2)	3 (2)
Lercanidipine	1 (1)	2 (1)
Thiazide	9 (6)	10 (7)
Clinical features		
Body mass index *(kg/m²)*	29.5 (4.6)	29.9 (5.2)
Weight *(kg)*	86.2 (14.6)	85.6 (15.7)
Waist-to-hip ratio Men	1.03 (0.09)	1.01 (0.07)
Women	0.93 (0.09)	0.93 (0.09)
Systolic blood pressure *(mmHg)*	159 (25)	160 (26)
Diastolic blood pressure *(mmHg)*	85 (11)	86 (12)
Daily dose of insulin *(IU/day)*	63 (26)	64 (30)
Laboratory variables		
Fasting plasma glucose *(mmol/l)*	10.3 (3.2)	10.0 (3.0)
Total cholesterol *(mmol/l)*	5.5 (1.3)	5.6 (1.1)
HDL cholesterol *(mmol/l)*	1.3 (0.4)	1.3 (0.4)
LDL cholesterol *(mmol/l)*	3.4 (1.0)	3.6 (1.0)
Triglycerides *(mmol/l)*	1.9 (1.5)	1.7 (1.1)
HbA_{1c} *(%)*	7.8 (1.2)	7.8 (1.2)

Data are given as mean (SD) or number (%).

The actual mean dose in the metformin-treated group was 2163 mg. Each patient maintained his/her maximally tolerated daily dose (1, 2 or 3 tablets of 850 mg) throughout the 16 weeks of treatment.

Markers of endothelial function

As compared to placebo, metformin treatment was associated with an increase in urinary albumin excretion of 21% ([-1 to +48]; p=0.06); a decrease in vWf of 6% ([-10 to -2]; p=0.0007); a decrease in sVCAM-1 of 4% ([-7 to -2]; p=0.0002); a decrease in sE-selectin of 6% ([-10 to -2]; p=0.008); a decrease in t-PA of 16% ([-20 to -12]; p<0.0001); and a decrease in PAI-1 of 20% ([-27 to -10]; p=0.0001) (Table 2).

Markers of inflammation

As compared to placebo, metformin treatment was associated with a decrease in CRP of 3% ([-16 to +12]; p=0.7) and a decrease in sICAM-1 of 2% ([-4 to +1]; p=0.3) (Table 2).

Markers of glycaemia and other variables

As compared to placebo, metformin treatment was associated with a decrease in HbA_{1c} of 8% ([-11 to -6]; p<0.0001); a decrease in fasting plasma glucose of 11% ([-15 to -8]; p<0.0001); a decrease in postprandial plasma glucose of 9% ([-13 to -5]; p<0.0001); a decrease in insulin dose of 8% ([-11 to -4]; p<0.0001); a decrease in BMI of 2% ([-3 to -1]; p=0.0006); and a decrease in LDL cholesterol concentration of 6% ([-9 to -3]; p=0.0003). LDL particle size and triglyceride concentrations did not change significantly during metformin or placebo treatment (Table 2).

Additional analyses

First, we carried out adjustment for age sex, menopausality, blood-pressure, use of ASA and antihypertensive medication, and for baseline values of the markers for endothelial function and inflammation to rule out that a non-significant imbalance in these variables at baseline among the two treatment groups might have affected the outcome. This did not materially change any of the results (data not shown). Second, to investigate whether metformin-associated changes in markers of endothelial function and inflammation were mediated by metformin-associated changes in other variables (i.e., HbA_{1c}, fasting or postprandial plasma glucose, insulin dose, BMI, LDL cholesterol concentration or particle size, and triglyceride concentration) we adjusted for changes in these variables in the analyses. This had no effect on the changes in vWf, sVCAM-1, t-PA, and PAI-1, CRP or sICAM-1. However, the difference in sE-selectin between the metformin and placebo group was in part (about 25%) dependent on the difference in fasting plasma glucose and triglyceride between the groups. In addition, the increase in urinary albumin excretion in the metformin group became statistically significant after adjustment for HbA1c (+ 32% [+7% to +63%] vs. placebo; p=0.01), which decreased under metformin treatment (-8% [-11 to -6] vs. placebo; p<0.0001), and this decrease in

Table 2. Markers of endothelial function and of inflammation at baseline and after 16 weeks of treatment with placebo or metformin

	Baseline (t_0)		16 weeks (t_1)		Change (%)			P
	Placebo (P)	Metformin (M)	Placebo (P)	Metformin (M)	P t_1 vs. t_0	M t_1 vs. t_0	M vs. P	M vs. P
Markers of endothelial function								
UAE *(mg/mmol)*	1.2 (0.9-1.5)	1.0 (0.8-1.3)	1.2 (1.0-1.6)	1.2 (1.0-1.6)	+4 (-11 to +20)	+26 (+11 to +43)	+21 (-1 to +48)	0.06
vWf *(%)*	136 (129-143)	143 (136-151)	137 (130-143)	135 (128-142)	0 (-3 to +4)	-6 (-9 to -3)	-6 (-10 to -2)	0.0007
sVCAM-1 *(ng/ml)*	879 (850-909)	919 (885-954)	883 (852-915)	878 (849-907)	0 (-2 to +2)	-4 (-7 to -2)	-4 (-7 to -2)	0.0002
sE-selectin *(ng/ml)*	47.3 (44.1-50.8)	51.0 (47.9-54.2)	47.9 (44.8-51.3)	48.6 (45.6-51.8)	+1 (-2 to +5)	-5 (-7 to -2)	-6 (-10 to -2)	0.008
t-PA *(ng/ml)*	11.8 (11.1-12.6)	12.7 (11.9-13.4)	12.0 (11.3-12.7)	10.7 (10.0-11.4)	+1 (-2 to +5)	-15 (-18 to -12)	-16 (-20 to -12)	<0.0001
PAI-1 *(ng/ml)*	95.0 (84.8-106)	94.5 (83.6-107)	98.8 (87.9-111)	79.4 (69.9-90.2)	+4 (-3 to +12)	-16 (-12 to -9)	-20 (-27 to -10)	0.0001
Markers of inflammation								
CRP *(mg/l)*	3.27 (2.80-3.81)	3.26 (2.80-3.78)	3.34 (2.89-3.86)	3.24 (2.80-3.75)	+2 (-8 to +14)	-1 (-10 to +10)	-3 (-16 to +12)	0.7
sICAM-1 *(ng/ml)*	604 (580-628)	614 (590-639)	601 (579-623)	602 (577-628)	0 (-3 to +2)	-2 (-4 to 0)	-2 (-4 to +1)	0.3
Markers of glycaemia and other variables								
HbA_{1c} *(% Hb)*	7.8 (7.6-8.0)	7.8 (7.6-7.9)	7.5 (7.4-7.7)	6.9 (6.7-7.0)	-3 (-5 to -2)	-11 (-13 to -10)	-8 (-11 to -6)	<0.0001
Fasting plasma glucose *(mmol/l)*	9.2 (8.9-9.5)	9.2 (8.9-9.5)	8.6 (8.4-8.8)	7.6 (7.4-7.8)	-7 (-9 to -5)	-17(-20 to -15)	-11 (-14 to -8)	<0.0001
Postprandial plasma glucose *(mmol/l)*	10.0 (9.7-10.3)	10.0 (9.7-10.4)	8.6 (8.4-8.9)	7.8 (7.6-8.0)	-14 (-16 to -11)	-22 (-25 to -19)	-10 (-19 to -6)	<0.0001
Insulin dose *(U/kg)*	0.7 (0.2-1.2)	0.7 (0.2-1.3)	0.8 (0.2-1.3)	0.7 (0.0-1.4)	+8 (-20 to +36)	0 (-23 to +33)	-8 (-11 to -4)	<0.0001
BMI *(kg/m²)*	29.6 (20.5-38.7)	29.6 (19.8-39.5)	30.0 (19.4-40.7)	29.5 (19.5-39.4)	+2 (-12 to +15)	-1 (-7 to +5)	-2 (-3 to -1)	0.0006
LDL cholesterol *(mmol/l)*	3.4 (3.3-3.6)	3.6 (3.4 –3.7)	3.4 (3.3-3.6)	3.4 (3.2-3.5)	0 (-2 to +2)	-6 (-8 to -3)	-6 (-9 to -3)	0.0003
LDL particle size *(nm)*	21.6 (21.5-21.7)	21.7 (21.6-21.8)	21.6 (21.5-21.7)	21.7 (21.6-21.8)	0 (0 to 0)	0 (0 to 0)	0 (0 to 0)	0.4
Triglycerides *(mmol/l)*	1.5 (1.3-1.6)	1.4 (1.2-1.5)	1.5 (1.4-1.7)	1.4 (1.2-1.5)	+3 (-4 to +10)	0 (-6 to +6)	-3 (-12 to +7)	0.6

Data at baseline and follow-up are presented as mean with 95% CI, or, when log-transformed, as geometric mean with 95% CI. Change is expressed as the mean percentage of change accompanied with a 95% CI. Reported p-values are the result of Student's t-tests. UAE, urinary albumin excretion; vWf, von Willebrand factor; sVCAM-1, soluble vascular cell adhesion molecule-1; t-PA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; CRP, C-reactive protein; sICAM-1, soluble intercellular adhesion molecule-1.

HbA1c was itself associated with a decrease in urinary albumin excretion (1.4% [1.0 to 1.9] per 1% HbA1c decrease).

Discussion

We found that, as compared to placebo, 16 weeks of metformin treatment in patients with type 2 diabetes intensively treated with insulin was associated with significant improvement of endothelial function but not of chronic, low-grade inflammation.

Type 2 diabetes is a state of generalised endothelial dysfunction, i.e. there is impairment of many endothelial functions, such as regulation of vasomotor tone, leukocyte adhesion, haemostasis and fibrinolysis, in many vascular beds.[20] Endothelial dysfunction in type 2 diabetes tends to be progressive and is strongly associated with cardiovascular disease risk.[4] We found that metformin treatment was associated with decreases in the plasma levels of vWf, sVCAM-1, sE-selectin, t-PA, and PAI-1, i.e. with improvement of the endothelial regulation of haemostasis (vWf), leukocyte adhesion (sE-selectin and sVCAM-1) and fibrinolysis (t-PA and PAI-1). For vWf, sE-selectin, t-PA and PAI-1, these findings are in accordance with previous experience in diabetic and non-diabetic individuals.[5, 6, 8, 24-26] Interestingly and importantly, changes in the plasma levels of these markers were independent of metformin-associated favourable changes in body weight, glycaemic control, insulin dose, and LDL cholesterol concentration, and they were also independent of LDL particle size, and triglyceride concentrations. The only exception was the change in plasma sE-selectin, which was in part explained by the metformin-associated improvement in glycaemic control and by triglyceride levels, in accordance with previous data on the role of glucose and lipids in the regulation of E-selectin synthesis.[27-29] In addition, changes in the plasma levels of the markers could not be attributed to the influence of the other variables studied. LDL particle size did not change upon treatment and therefore, the decrease in LDL cholesterol concentration could not in any part be explained by decreasing LDL particle size, a phenomenon which has been associated with an increased cardiovascular risk.[30] Smoking habits did not differ between the two groups at baseline with respect to the number of cigarettes smoked per day (M 2.1, P 3.3;), nor with respect to the number of patients who quit smoking in the 16-week follow-up period. The number of smokers was somewhat more unbalanced between the groups (M 31 (21%); P 50 (31%)). This baseline difference, however, has not led to any difference in vascular function at baseline, as measured by the markers of endothelial function and inflammation. No differences in blood pressure existed at baseline or after 16 weeks of treatment with metformin, as published previously.[14] No difference in alcoholic consumption existed between the two groups. Data on adherence to lifestyle were not available. Taken together, these findings raise the possibility that improvement of endothelial function by metformin may represent a largely glucose-independent pathway through which metformin decreases risk of cardiovascular disease in type 2 diabetes.[1]

An important assumption in this reasoning is that plasma levels of these markers are valid indicators of endothelial function. This, in turn, requires that

endothelial cells are the major source of the plasma concentrations of these proteins, and that protein concentration is determined by synthesis rather than by clearance. The validity of these assumptions is uncertain.[20] Only sE-selectin and t-PA are synthesised exclusively by endothelial cells. However, t-PA in plasma binds to PAI-1, and t-PA concentrations may mainly reflect the concentration of PAI-1, which is synthesised not only by endothelial cells, but also by hepatocytes and adipocytes. In addition, there is virtually no information on the regulation of the clearance of these proteins in type 2 diabetes, except for vWf, for which there is indirect evidence that its plasma concentration, in type 2 diabetes, is determined by synthesis rather than clearance.[31]

Unexpectedly, urinary albumin excretion tended to increase slightly during short-term treatment with metformin, an increase that was statistically significant after adjustment for changes in HbA1c. Notably, a decrease in HbA1c, as expected, was itself associated with a decrease of urinary albumin excretion. Taken together, these results suggest that metformin can decrease urinary albumin excretion by improving glycaemic control, while at the same time it can increase urinary albumin excretion through other mechanisms. Previous studies of the effect of metformin on urinary albumin excretion showed either no effect[32, 33] or a decrease.[8, 34] Nevertheless, our results are unexpected, and we cannot exclude that they represent a chance finding. In addition, even if the findings are valid, their interpretation is unclear. Small increases in urinary albumin excretion, i.e. microalbuminuria, are strongly associated with endothelial dysfunction and have been postulated to reflect increased endothelial permeability to macromolecules.[35] This concept may not hold under all circumstances, however, because urinary albumin excretion depends on glomerular albumin permeation (i.e. pressure, permeability, and surface area) and tubular reabsorption. The discrepancy between the effects of metformin on urinary albumin excretion (unfavourable) and those on the plasma markers of endothelial function (favourable) raises the possibility that the effect of metformin on urinary albumin excretion may be unrelated to endothelial function. These possibilities require further investigation.

We found no effect of short-term treatment with metformin on markers of low-grade inflammation, i.e. sICAM-1 and CRP. To the best of our knowledge, no placebo-controlled trials designed to study the effect of metformin on sICAM-1 or CRP in diabetes have been reported. However, a study in women with polycystic ovary syndrome (PCOS) reported a decrease in CRP after metformin treatment.[36] The differences between diabetic and PCOS patients require further investigation.

We showed that the effects of metformin on endothelial function were mostly unrelated to decreases in hyperglycaemia, insulin dose, and BMI, suggesting that metformin may have direct effects on the endothelium.[37-39] However, we cannot exclude that metformin improves endothelial function by decreasing advanced glycation endproduct levels,[40-42] by altering the secretion of adipocyte-derived mediators (such as free fatty acids, leptin, resistin, and adiponectin),[43-49] by decreasing inflammatory activity in ways not reflected by CRP and sICAM-1,[50, 51] and (or) by improving insulin sensitivity, of which a change in insulin dose may be an insufficiently accurate marker. These possibilities require further study.

We conclude that, in patients with type 2 diabetes treated with insulin, 16 weeks of metformin treatment, as compared to placebo, was associated with improvements in plasma markers of endothelial function, which were mostly unrelated to changes in HbA_{1c}, fasting or postprandial plasma glucose, insulin dose, BMI, LDL cholesterol, and triglyceride concentrations. Metformin may thus have specific effects on endothelial function, which may explain, in part, why metformin appears to be associated with a decreased risk of cardiovascular disease in type 2 diabetes. This hypothesis requires further testing.

Conflict of Interest Statement

Adriaan Kooy, as principal investigator, received grants to support the HOME-study from Byk, Lifescan, Merck-Santé, Merck, Sharpe & Dohme, and Novo Nordisk. Philippe Lehert is an occasional consultant for Merck-Santé. Daniel Bets is an employer of Merck B.V., responsible as trial monitor for the proper conduct of the study. For the other authors no conflict of interest exists.

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Chapter 4

Long-term effects of metformin on metabolism and micro- and macrovascular disease in patients with type 2 diabetes

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Abstract

Background

We investigated whether metformin has sustained beneficial metabolic and (cardio)vascular effects in patients with type 2 diabetes (DM2).

Methods

We studied 390 patients treated with insulin in the outpatient clinics of three hospitals in a randomized placebo-controlled trial with a follow-up of 4.3 years. Either 850 mg metformin or placebo (1-3 times daily) was added to insulin therapy. The primary endpoint was an aggregate of micro- and macrovascular morbidity and mortality. Secondary endpoints were microvascular and macrovascular morbidity and mortality, as separate aggregate scores. In addition, effects on HbA1c, insulin requirement, lipids, blood pressure, body weight, and BMI were analyzed.

Results

Metformin treatment prevented weight gain (-3.07 kg [-3.85 to -2.28]; p<0.001), improved glycemic control (HbA1c: -0.4%point [-0.55 to -0.25]; p<0.001) – despite the aim of similar glycemic control in both groups –, and reduced insulin requirements (-19.63 IU/day [-24.91 to -14.36]; p<0.001). Metformin was not associated with an improvement in the primary endpoint. It was, however, associated with an improvement in the secondary, macrovascular endpoint (HR 0.61 (0.40 to 0.94; p=0.02), which was partly explained by the difference in weight. The NNT to prevent one macrovascular endpoint was 16.1 (9.2 to 66.6).

Conclusion

Metformin, added to insulin in patients with DM2, improved body weight, glycemic control and insulin requirements, but did not improve the primary endpoint. Metformin did, however, reduce the risk of macrovascular disease after a follow-up of 4.3 years. These sustained beneficial effects support the policy to continue metformin after the introduction of insulin in any patient with DM2, unless contraindicated.

Introduction

The rising incidence of type 2 diabetes (DM2) makes diabetes an increasingly important cause of cardiovascular disease and death. Up to 75% of patients with DM2 will die of a cardiovascular complication.[1] Therefore, prevention of cardiovascular complications in DM2 is crucial.

Questions remain concerning the beneficial effects of metformin in DM2. The only randomized intervention trial on this subject was the UKPDS, the results of which suggest a cardioprotective role for metformin. However, the design and analyses of the UKPDS have raised considerable debate.[2] Nevertheless, data from cohort studies support the results from the UKPDS,[3, 4] but a need for clinical, randomized intervention trials still exists to help clarify this issue. In addition, the mechanisms through which metformin may decrease the risk of micro- and macrovascular disease are unclear and may include reduction of weight gain and hyperinsulinemia, improvement of endothelial function and fibrinolysis, and reduction of low grade inflammation, oxidative stress and glycation.[5-7]

DM2 is a progressive disease, and many patients will need treatment with insulin during the course of the disease.[8] Several short-term studies in insulin-treated DM2 patients have shown that metformin can improve glycemic control and reduce insulin requirements and weight gain.[9-11] It has not been studied whether metformin has long-term beneficial effects in such patients.

We hypothesized that, in patients with DM2 treated with insulin, metformin, as compared to placebo, will have sustained beneficial metabolic effects, even at the same level of glycemic control, and thus decrease (cardio)vascular disease. We designed the randomized, placebo-controlled, multicenter trial “Hyperinsulinemia: the Outcome of its Metabolic Effects” (HOME) to investigate these issues during a planned follow-up of 4.3 years.[11]

Methods

Patients

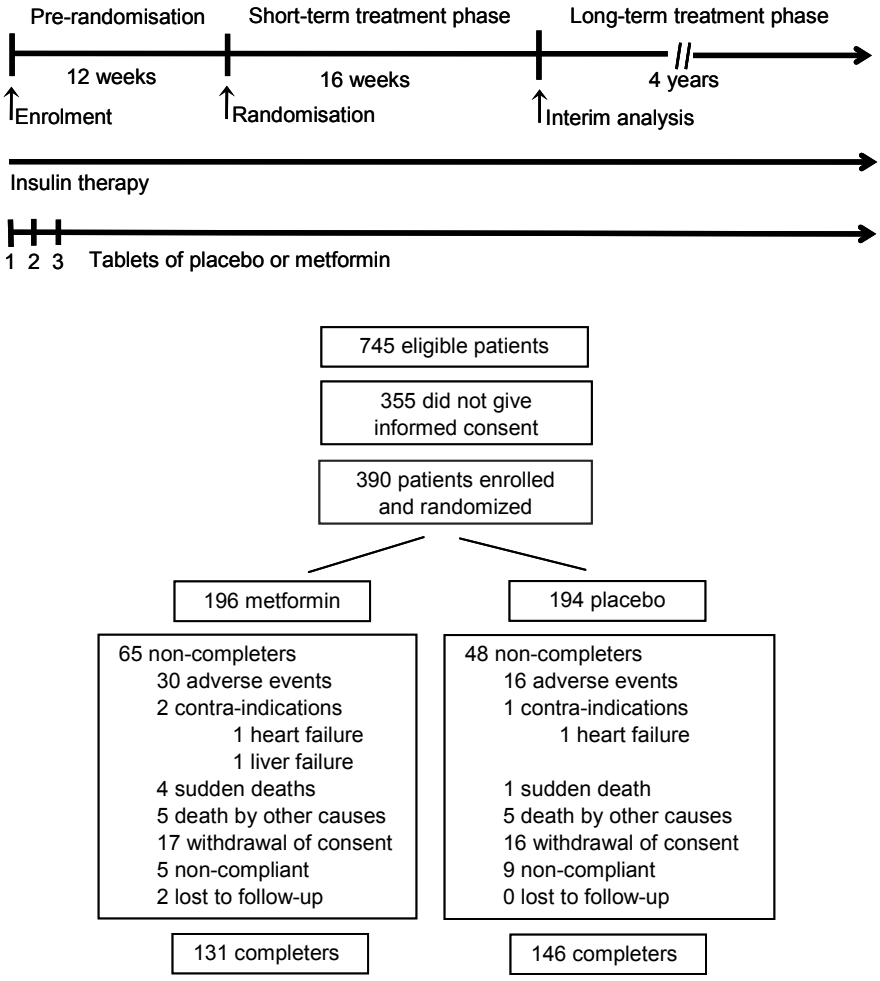
We included 390 patients with type 2 diabetes (age 30-80 years) as previously described.[11] All patients gave written informed consent. The medical ethical committees of the three participating hospitals approved the trial protocol. The trial has been conducted in accordance with the Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) dated 17 July 1996, and in accordance with the Declaration of Helsinki (revised version of Hong Kong in 1989 and Edinburgh in 2000).

Study design

The HOME trial was conducted in the outpatient clinics of three hospitals (Hoogeveen, Meppel, and Coevorden; The Netherlands). Patients were randomly allocated to either placebo or metformin by aid of a computer program, which allocated a random number

to identical looking boxes of either metformin or placebo. The trial design consisted of three phases, as shown in Figure 1.[11]

Figure 1. HOME trial profile and flow chart



Glycemic control and insulin titration

In the pre-randomisation phase, we aimed to optimise glycemic control by intensive glucose monitoring and insulin adjustments (target glucose levels, fasting: 4-7 mmol/l, and postprandial: 4-10 mmol/l). All subjects monitored their glucose levels at home every two weeks (i.e. just before and ~90 minutes after breakfast, lunch, and dinner, and at bedtime) using the same monitoring device (Glucotouch; Lifescan, Beerse,

Belgium). Individual insulin titration took place according to good clinical practice to reach the target glucose levels and to prevent hypoglycemia. If the target values for glycemic control were difficult to reach, the study nurse consulted the principal investigator for advice to optimise the insulin therapy. This glucose-monitoring and insulin adjustment scheme was continued during the whole trial, as detailed elsewhere.[7, 11]

Clinical endpoints (Table 1)

The study was designed to study the effects of metformin on metabolic and disease-related endpoints, as previously described.[11] The metabolic endpoints examined were body weight, BMI, waist-to-hip ratio (WHR), HbA1c , home-monitored concentrations of pre- and postprandial glucose, fasting plasma insulin, and the daily dose of insulin. We also collected data on blood pressure and plasma lipids. In addition, we collected information on the incidence of hypoglycemic events. A hypoglycemic event was defined as a blood glucose concentration below 3.8 mmol/l with symptoms of hypoglycemia or a value below 3.0 mmol/l with or without such symptoms. Data on weight, HbA1c, home-monitored concentrations of pre- and postprandial glucose, and blood pressure were obtained each visit (every three months). Waist-to-hip ratio was measured at baseline, after four, seven, and 48 months. Plasma lipids were measured at baseline and every six months thereafter; plasma insulin was measured at baseline, after four months and every 12 months thereafter.

On the assumption that several shared determinants of macro- and microvascular disease (such as hypertension, obesity, endothelial dysfunction, and chronic inflammation[12-14]) might be influenced by metformin[5, 7], the primary endpoint was constructed as a combination of macro- and microvascular disease. Secondary disease-related endpoints were macrovascular and microvascular disease separately (Table 1). Before disclosure of the treatment codes an independent scientific committee (one specialist in vascular medicine and one specialist in endocrinology) checked the (non) registration of all disease-related endpoints. No misreporting was found.

In order to optimally study the metabolic endpoints, patients continued participation in the trial after the occurrence of a non-fatal disease-related endpoint, unless a contra-indication for metformin emerged. Therefore, a patient who completed the planned 4.3 years of follow-up (a 'completer'), may or may not have encountered a disease-related endpoint. A non-completer could thus have been withdrawn before the final visit for a variety of reasons: adverse effects, withdrawal of consent, loss to follow-up, a fatal endpoint, or because of the development of a contra-indication to metformin, notably renal or heart failure (New York Heart Association class III/IV). We screened for renal failure by monitoring creatinine clearance. Patients with a creatinine clearance between 60 and 40 ml/min were allowed a maximum of two tablets a day; those between 40 and 30 ml/min one tablet a day; and those below 30 ml/min were withdrawn from the study.

Table 1. Metabolic and disease-related endpoints of the HOME-trial

Endpoints	
The metabolic endpoints	Body weight, BMI, waist-to-hip ratio, plasma HbA_{1c} concentration, home-monitored concentrations of pre- and postprandial glucose, plasma insulin concentration, daily dose of insulin, blood pressure, plasma lipids
The primary disease-related endpoint	Clinical events 1 – 16
The secondary disease-related endpoints	I. Macrovascular: clinical events 1 – 13 II. Microvascular: clinical events 14 – 16

Clinical events		Definition		
1	Myocardial infarction	Documented diagnosis by a cardiologist		
2	Heart failure	Documented diagnosis by a cardiologist		
3	Changes of EKG	Minnesota scores 1.1-1.3, 4.1-4.3, 5.1-5.3, 7.1		
4	Acute coronary syndrome	Documented diagnosis by a cardiologist; having resulted in hospital admission		
5	Diabetic foot	Documented diagnosis by an internist and/or surgeon; having resulted in hospital admission		
6	Stroke	Documented diagnosis by a neurologist		
7	Transient ischemic attack	Documented diagnosis by a neurologist		
8	Peripheral arterial disease	Diagnosed by angiography		
9	Peripheral arterial reconstruction	Determined by a physician and well documented in the original medical record and in the CRF		
10	PTCA	Determined by a physician and well documented in the original medical record and in the CRF		
11	CABG	Determined by a physician and well documented in the original medical record and in the CRF		
12	Non-traumatic amputation	Determined by a physician and well documented in the original medical record and in the CRF		
13	Sudden death	Determined by a physician and well documented in the original medical record and in the CRF		
14	Progression of retinopathy A→C or B→C	Classified by an ophthalmologist: A. None B. Non-proliferative C. Proliferative		
15	Progression of nephropathy A→B or A→C or B→C	Expressed as albuminuria (albumin-to-creatinine ratio in urine, A/C in [mg/mmol])		
			Men	Women
		A = normal	A/C < 2.5	A/C < 3.5
		B = microalbuminuria	2.5 < A/C < 25	3.5 < A/C < 35
		C = macroalbuminuria	> 25	> 35
16	Progression of neuropathy (A→B or A→C or A→D or B→C or B→D or C→D, with a difference in score of at least 6 points)	Diabetic polyneuropathy was evaluated by constructing a neuropathy score [a]		
		A = normal, none	0 points	
		B = mild	1-9 points	
		C = moderate	10-18 points	
		D = severe	19-33 points	
17	Death by other cause	Noncardiovascular nonsudden death		

Abbreviations: CABG, coronary artery bypass graft; CRF, clinical research file; ECG, electrocardiogram; HOME, Hyperinsulinemia: the Outcome of its Metabolic Effects; PTCA, percutaneous transluminal coronary angioplasty.
[a] See Valk et al [39]

Statistical analyses

Sample size and power analysis

The planned study sample size of 390 patients was based on an expected difference in the occurrence of the primary endpoint of at least 8%-points between the treatment groups after 4.3 years, with an expected incidence of 20% in the placebo group and 12% in the metformin group (one-tailed test on Proportional Hazard regression with α = 0.05 and β = 0.25).

Data analysis

The data presented concern all randomized patients (intention-to-treat (ITT) sample; n=390). 95% confidence intervals are used; p-values for the individual variables represent two-sided tests.

The effects of metformin on the metabolic endpoints were assessed by analysing the complete time course for each variable. For this purpose, the summary mean (mean score of non-missing values over the entire observation period) constitutes the appropriate repeated measurement technique.[15] This summary mean was then compared between the treatment groups through an analysis of covariance (ANCOVA), while adjusting for baseline values, and baseline differences in age, gender, smoking, and the prior occurrence and severity of cardiovascular disease. The chi-square test was used to compare the number of hypoglycemic events between the two treatment groups.

The effects of metformin on the disease-related endpoints were assessed by comparing time to first event by means of Proportional Hazards multiple regression analyses. This was the primary analysis for both the primary and the secondary endpoints. Because of the baseline differences in age, gender, smoking, statin use and the prior occurrence and severity of cardiovascular disease between the two treatment groups, we adjusted for these variables in all analyses. As an additional analysis, Multiple Endpoint survival technique was used to take into account multiple events.[16] The severity of the cardiovascular history at baseline was computed as the sum score of cardiovascular events as follows: myocardial infarction absent=0, present=1; cardiovascular intervention (peripheral arterial reconstruction, percutaneous transluminal coronary angioplasty, and coronary arterial bypass graft) absent=0, present=1; transient ischemic attack absent=0, present=1; stroke absent=0, present=1; dyspnea NYHA class no=0, I=1, II=2, III=3, IV=4; angina pectoris NYHA no=0, I=1, II=2, III=3, IV=4; intermittent claudication no=0, >100m=1, 50-100m=2, <50m=3, rest=4; and amputation absent=0, present=1.

To assess to what extent the metformin-associated changes in the disease-related endpoints could be explained by the metformin-associated changes in the metabolic endpoints, we used proportional hazards multiple regression analysis to re-analyze the HRs after adjusting for metabolic variables. Main effects and First order Interaction with treatments were tested and non significant effects (p<.05) were sequentially removed to reduce type I error and promote stability of the model.

Table 2. Baseline Characteristics (Intention to Treat Sample)

		Placebo (n=194)	Metformin (n=196)
Demography			
Men/women		97/97	81/115
Age *(years)*		59 (11)	64 (10)
Currently smoking *n (%)*		59 (30)	38 (19)
Duration of diabetes *(years)*		12 (8)	14 (9)
Insulin treatment *(years)*		6 (6)	7 (8)
Concomitant medication			
Lipid-lowering drugs *n (%)*		31 (16)	32 (16)
Blood-pressure-lowering drugs *n (%)*		75 (39)	93 (47)
Metabolic variables			
Weight *(kg)*		87 (15)	85 (16)
Body mass index *(kg/m^2)*		30 (5)	30 (5)
Waist-to-hip ratio	Men	1.03 (0.1)	1.02 (0.1)
	Women	0.93 (0.1)	0.92 (0.1)
Plasma HbA1c *(%)*		7.9 (1.2)	7.9 (1.2)
Preprandial glucose *(mmol/l)*		8.8 (1.8)	8.6 (1.8)
Postprandial glucose *(mmol/l)*		10.2 (2.0)	10.2 (2.1)
Plasma insulin *(pmol/l)*		301 (686)	248 (545)
Daily dose of insulin *(IU/day)*		64 (25)	62 (29)
Systolic blood pressure *(mmHg)*		159 (25)	160 (25)
Diastolic blood pressure *(mmHg)*		86 (11)	86 (12)
Total cholesterol *(mmol/l)*		5.5 (1.2)	5.6 (1.3)
LDL cholesterol *(mmol/l)*		3.4 (1.0)	3.6 (1.1)
Triglycerides *(mmol/l)*		1.9 (1.5)	1.7 (1.2)
HDL cholesterol *(mmol/l)*		1.3 (0.4)	1.3 (0.4)
Prior macro- and microvascular disease			
Cardiovascular history		0.92 (1.3)	1.17 (1.4)
Diabetic polyneuropathy score		7.51 (5.4)	8.36 (6.3)

Data are given as mean (SD) except where indicated otherwise

Results

Patients

We screened the medical files of all three participating outpatient clinics and identified 745 eligible patients (Figure 1). All were approached to enroll into the trial and 390 subjects gave written informed consent. 196 subjects were randomized to receive metformin (M) and 194 to receive placebo (P). Out of 390 included patients, 277 subjects (= 72%) completed the HOME trial (completers). Of the non-completers, 46 experienced adverse events (30 M, 16 P), three patients developed a contra-indication to metformin (one liver failure [M], two heart failure [one M, one P]), 15 encountered a fatal disease-related endpoint (nine M, six P), 47 withdrew their consent (22 M, 25 P), and two patients were lost to follow-up (two M). Of the 46 patients with adverse events,

33 experienced diarrhoea (22 M, 11 P), 20 flatulence (ten M, ten P), 15 fatigue (seven M, eight P), 14 pruritus (five M, nine P), 15 headaches (six M, nine P), 16 heartburn (seven M, nine P), and 20 nausea (ten M, ten P). Non-completers did not differ from completers with respect to duration of diabetes, prior occurrence and severity of cardiovascular disease, age, or weight. More women than men were non-completers (male:female 1:1.8 vs 1:1, p=0.011).

Table 2 shows baseline characteristics of all randomized patients. Patients randomized to metformin were slightly older than patients randomized to placebo (63.6 ± 9.6 vs. 59.1 ± 11.0 years, respectively), and had a more extensive cardiovascular history (1.17 vs. 0.92, respectively), and were less often smokers. The other characteristics were comparable between the treatment groups. The actual mean dose in the metformin group in the short-term treatment phase was 2163 mg, and 2050 mg in the long-term treatment phase. Although the use of statins was low at baseline, in accordance with treatment guidelines at the start of the study, its use increased to 67 patients in the metformin and 54 patients in the placebo group at the final visit.

Table 3. Overview of the main endpoints: I. Metabolic endpoints

		Placebo (n=194)			Metformin (n=196)		
		Baseline	Last visit	Summary mean	Baseline	Last visit	Summary mean
Body weight *(kg)*		87 (15)	91 (17)	90 (16)	85 (16)	87 (17)	85 (16)
Body Mass Index *(kg/m^2)*		30 (5)	31 (5)	31 (5)	30 (5)	30 (5)	30 (5)
Waist-to-hip ratio	Men	1.03 (0.1)	1.03 (0.1)	1.03 (0.1)	1.02 (0.1)	1.03 (0.1)	1.03 (0.1)
	Women	0.93 (0.1)	0.95 (0.1)	0.94 (0.1)	0.92 (0.1)	0.93 (0.1)	0.93 (0.1)
Plasma HbA1c *(%)*		7.9 (1.2)	7.9 (1.1)	7.9 (1.0)	7.9 (1.2)	7.7 (1.1)	7.5 (1.0)
Preprandial glucose *(mmol/l)*		8.8 (1.8)	8.0 (1.5)	8.2 (1.6)	8.6 (1.8)	8.1 (1.6)	7.8 (1.3)
Postprandial glucose *(mmol/l)*		10.2 (2.0)	9.0 (1.6)	9.4 (1.3)	10.2 (2.1)	8.9 (1.9)	8.9 (1.5)
Plasma insulin *(pmol/l)*		301 (686)	522 (1068)	484 (961)	248 (545)	323 (598)	314 (641)
Daily dose of insulin *(IU/day)*		64 (25)	100 (59)	84 (40)	62 (29)	75 (50)	67 (40)
SBP *(mmHg)*		159 (25)	141 (14)	154 (17)	160 (25)	141 (18)	153 (18)
DBP *(mmHg)*		86 (11)	79 (10)	85 (9)	86 (12)	77 (11)	85 (9)
Total cholesterol *(mmol/l)*		5.5 (1.2)	4.2 (0.8)	4.7 (0.9)	5.6 (1.3)	4.2 (0.7)	4.8 (1.1)
LDL cholesterol *(mmol/l)*		3.4 (1.0)	2.2 (0.6)	2.7 (0.8)	3.6 (1.1)	2.1 (0.6)	2.8 (0.9)
Triglycerides *(mmol/l)*		1.9 (1.5)	1.6 (1.6)	1.7 (1.2)	1.7 (1.2)	1.5 (0.9)	1.6 (1.0)
HDL cholesterol *(mmol/l)*		1.25 (0.4)	1.33 (0.4)	1.27 (0.4)	1.3 (0.4)	1.35 (0.4)	1.32 (0.4)

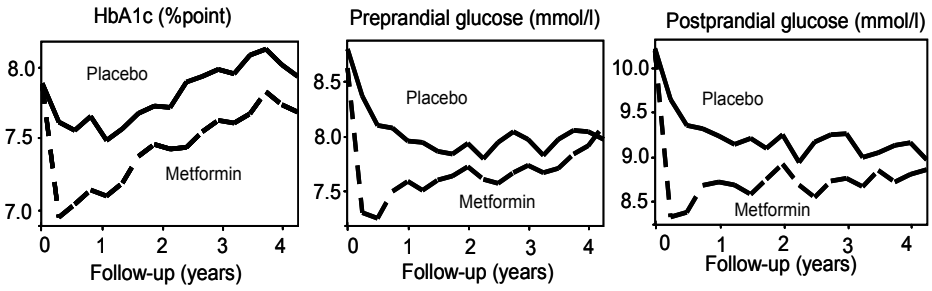
Data are given as mean (SD) (%). SBP=systolic blood pressure; DBP=diastolic blood pressure. Differences (with p-values) between metformin and placebo are described in more detail in text and in Figure 2 and 3

Metabolic endpoints (Table 3)

Metformin improved glycemic control and reduced insulin requirements (Figure 2, 3)

Despite the aim of similar glycemic control in both groups, after 4.3 years of treatment, the difference in the summary mean for HbA1c between metformin and placebo was -0.40%-point (-0.55 to -0.25; p<0.001). For home-monitored concentrations of pre- and postprandial glucose, the differences were -0.29 mmol/l (-0.51 to -0.07; p=0.01) and -0.44 mmol/l (-0.68 to -0.20; p<0.01), respectively. For plasma insulin, the difference was -124 pmol/l (-231 to -16; p=0.02). For the daily dose of insulin, the difference was -19.63 IU/day (-24.91 to -14.36; p< 0.001) or -0.18 IU/kg (-0.23 to -0.12; p<0.001).

Figure 2. Glycaemic control: HbA1c, pre- and postprandial glucose values



Despite the aim of similar glycaemic control in both groups, the summary means were significantly different between the groups for HbA1c (p<0.001), home-monitored pre-prandial glucose (p=0.01) and home-monitored postprandial glucose (p<0.01)

Metformin prevented weight gain (Figure 3)

After 4.3 years of treatment, the difference in the summary mean for body weight between metformin and placebo was -3.07 kg (-3.85 to -2.28; p<0.001). The difference for BMI was -1.09 kg/m^2 (-1.37 to -0.81; p<0.001). The difference for the waist-to-hip ratio was -0.015 (-0.029 to -0.001; p=0.04).

Blood pressure and lipid profile

Metformin did not decrease blood pressure

After 4.3 years of treatment, the difference in the summary mean between metformin and placebo was -0.51 mmHg (-2.76 to 1.44; p=0.38) for systolic and -0.88 mmHg (-3.21 to 1.45; p=0.46) for diastolic blood pressure.

Metformin treatment did not improve the plasma lipid profile

After 4.3 years of treatment, the difference in the summary mean between metformin and placebo was 0.02 mmol/l (-0.12 to 0.16; p=0.78) for total cholesterol; 0.00 mmol/l (-0.12 to 0.12; p=0.98) for LDL cholesterol; 0.01 mmol/l (-0.11 to 0.14; p=0.82) for

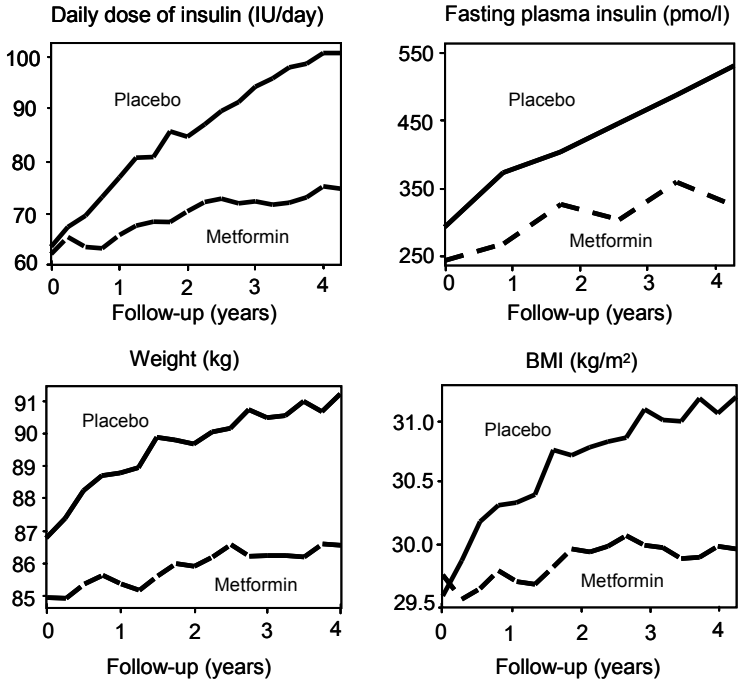
triglycerides; and 0.00 mmol/l (-3.21 to 1.45; p=0.91) for HDL cholesterol. The use of lipid lowering drugs did not significantly differ between the groups, and adjustment for use of lipid-lowering drugs did not change the results.

Hypoglycemic events

Metformin did not increase the number of hypoglycemic events

After 4.3 years of treatment, no difference in the number of hypoglycemic events between metformin and placebo existed M 2.1 vs. P 2.6 total hypoglycemic events per person per year; p=0.89. In 0.3 and 0.3 events per person per year, respectively, help from others was needed; p=0.33.

Figure 3. Insulin requirements and weight



The summary means were significantly different between the groups for daily dose of insulin ($p < 0.001$) and fasting plasma insulin ($p=0.02$), for body weight ($p<0.001$), and BMI ($p<0.001$).

Disease-related endpoints (Table 4)

Metformin did not decrease the risk of the primary endpoint (Figure 4)

The unadjusted event rates were 28% for patients in the placebo and 31% in the metformin group. After adjustment for age, gender, smoking, and cardiovascular history,

the HR for the primary endpoint was 0.92 (0.72 to 1.18; p=0.33), and, if combined with death by other causes, 0.94 (0.74 to 1.19; p=0.37).

Table 4. Overview of the main endpoints: II. Disease-related endpoints

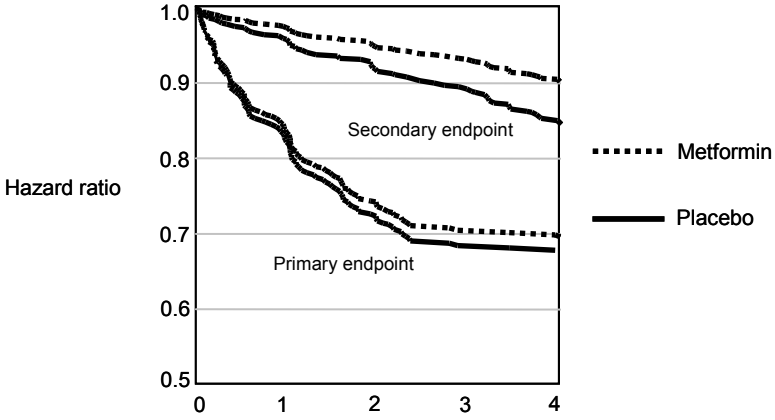
	Placebo (n=194)		Metformin (n=196)	
	Baseline	Last visit	Baseline	Last visit
Myocardial infarction	21 (11)	25 (13)	24 (12)	28 (14)
Heart failure	0	4 (2)	0	3 (2)
Ischemic changes of ECG	NA	14 (7)	NA	10 (5)
Acute coronary syndrome	0	7 (4)	4 (2)	6 (3)
Diabetic foot	6 (3)	11 (6)	6 (3)	9 (5)
Stroke	8 (4)	9 (5)	8 (4)	9 (5)
Transient ischemic attack	10 (5)	12 (6)	8 (4)	10 (5)
Peripheral arterial disease	10 (5)	18 (9)	14 (7)	21 (11)
Cardiovascular intervention	18 (9)	27 (14)	27 (14)	34 (17)
Non-traumatic amputation	3 (2)	4 (2)	5 (3)	7 (4)
Sudden death	NA	1 (1) *	NA	4 (2) ‡
Progression of retinopathy	NA	0	NA	1 (1)
Progression of nephropathy	NA	14 (7)	NA	15 (8)
Progression of neuropathy	NA	18 (9)	NA	19 (10)
Death by other causes	NA	5 (3)	NA	5 (3)

Data are given as number (%). Differences (with p-values) between metformin and placebo are described in more detail in text and in Figure 4
Abbreviations: ECG, electrocardiogram; NA, not applicable; * cardiovascular death; ‡ three cardiovascular deaths and 1 fatal car accident

Metformin decreased the risk of the secondary, macrovascular endpoint (Figure 4)
The unadjusted event rates were 18% for patients in the placebo and 15% in the metformin group. After adjustment for age, gender, smoking, and cardiovascular history, the HR for the secondary, macrovascular endpoint was 0.60 (0.40 to 0.92; p=0.04). The absolute risk difference between the groups was -6.1% (-10.5 to -1.5%; p=0.04), resulting in a number-needed-to-treat (NNT) to prevent one macrovascular endpoint of 16 (9 to 67). Exclusion of sudden death from the secondary, macrovascular endpoint did not change the results; HR 0.61 (0.40 to 0.94; p=0.02). Figure 4 shows the survival functions for the two treatment groups.

Metformin did not decrease the risk of the secondary, microvascular endpoint
The unadjusted event rates were 15% for patients in the placebo and 17% in the metformin group. After adjustment for age, gender, smoking, and prior diabetic polyneuropathy, the HR for the secondary, microvascular endpoint was 1.04 (0.75 to 1.44; p=0.43).

Figure 4. Survival functions for the primary and the secondary, macrovascular endpoint



Metformin treatment was not associated with an improvement in the primary endpoint. It was, however, associated with a decreased risk of the secondary, macrovascular endpoint (hazard ratio 0.61 [0.40 to 0.94; p=0.02]). NNT to prevent one macrovascular endpoint was 16 (9 to 67)

Additional analyses

After adjusting for the change in weight, the HR for the secondary, macrovascular endpoint was 0.77 (0.55 to 1.09; p=0.334) compared to 0.60 (0.40 to 0.92; p=0.04) without adjusting for change in weight. However, adjustments for the metformin-associated changes in other metabolic efficacy variables, such as blood pressure or lipid profile did not materially change our results. For example, after adjusting for changes in HbA1c, daily dose of insulin, and systolic blood pressure, the HR for the secondary, macrovascular endpoint was 0.34 (0.21 to 0.56; p=0.001), as compared to 0.60 (0.40 to 0.92; p=0.04) without such adjustments. The HR for the secondary macrovascular endpoint without including diabetic foot was 0.60 (0.44 to 0.85; p=0.05) compared to 0.60 (0.40 to 0.92; p=0.04).There was no interaction between hospital center and treatment, and adjustment for treatment center did not change any of the results. Analyses using the PP population showed similar results, although slightly more in favour of metformin, as compared to the ITT population. Analysis of the metabolic endpoints using last observation carried forward instead of the summary mean yielded very similar results.

There was loss of glycemic control over time (Figure 2), which was similar in both groups. 14% and 16% of patients in the placebo and metformin group, respectively, had a HbA1c of less than 7% at the final visit, and in both groups 14% of patients had a HbA1c of over 8% at the final visit, while having a HbA1c under 7% at baseline. Conversely, 10% and 9% patients in the placebo and metformin group,

respectively, had a HbA1c less than 7% at the final visit, while having a HbA1c over 8% at baseline.

Discussion

Our study on the effects of long-term metformin treatment in patients with DM2 treated with insulin had two main findings. First, metformin treatment was associated with beneficial effects on outcomes such as body weight and insulin requirements, and moderately beneficial effects on glycemic control (despite the aim of similar glycemic control in both groups), but not on blood pressure and the plasma lipid profile. Second, metformin treatment did not decrease the risk of the primary endpoint, but did decrease the risk of the secondary, macrovascular endpoint.

The favourable effects of metformin treatment on weight gain and insulin requirements are in accordance with previous findings from short-term studies.[9-11] The absence of a blood pressure-lowering effect of metformin is also consistent with previous studies and meta-analyses.[17-20] Previous short-term studies on plasma lipids have either shown no or only a small effect of metformin treatment.[18, 20, 21] Importantly, our study shows that the beneficial effects on weight gain and insulin requirements persist during 4.3 years of follow-up. The reductions of the daily dose of insulin and of plasma insulin levels seem to indicate lower insulin exposure levels in the metformin group.

The beneficial effects of metformin on weight and on insulin requirements continued to improve during 4.3 years of treatment. The improvements in glycemic control, however, occurred rapidly, but were not maintained throughout the long treatment period. The rate of the loss of glycemic control (approximately 1%-point HbA1c increase in 5 years) was similar to that seen in the UKPDS. It is unclear why this loss of glycemic control, which was comparable between the treatment groups, occurred. Insulin adjustments were made based on a treatment protocol, using target glucose values. Glycemic control deterioration after initial improvement has been frequently described before; a higher risk of glycemic relapse has been associated with insulin treatment, longer duration of diabetes, and weight gain.[22] In addition, glycemic relapse has been, at least partly, attributed to the progressive nature of DM2.[23] Indeed, in our study higher insulin dosages were needed over time, especially in the placebo group. A reluctance of both physician and patient to increase the insulin dosage in the face of deteriorating glycemic control has been described as a strong contributor to the deterioration of glycemic control.[24, 25] Our study nurses, however, tightly performed the protocol for glycemic control during the whole period of follow-up.

Overall, glycemic control was better in the metformin group, despite the aim of similar glycemic control in both groups. These data suggest that, in DM2 patients treated with insulin, metformin may affect glucose metabolism by improving the hepatic responsiveness to insulin, and by increasing the release of GLP-1.[26, 27]

To the best of our knowledge, no randomized, placebo-controlled trials on the effects of metformin on macro- or microvascular disease in insulin-treated DM2 patients

have been reported. In our study, metformin treatment was associated only with favourable effects on macrovascular disease and, somewhat unexpectedly, not on microvascular disease. Several reasons may account for this. In studies that have shown improved microvascular outcomes in treated diabetic patients, larger HbA1c differences were maintained over longer periods of time.[23, 28] In addition, improvements in blood pressure have been shown to be a important contributor to a decrease in microvascular disease risk.[29, 30] Thus, the small effect of metformin on glycemic control and the lack of an effect on blood pressure may explain why microvascular outcomes were not improved within the follow-up period of 4.3 years. The absence of a treatment effect on microvascular disease may explain why significant changes were not observed in the primary endpoint, in which microvascular events were incorporated.

In our study, the favourable effects of metformin on macrovascular disease could be partly explained (roughly 40%) by the metformin-associated change in weight. All other metformin associated changes in metabolic or hemodynamic variables, such as HbA1C and daily dose of insulin, did not seem to contribute to the favourable effect of metformin on macrovascular disease. Taken together, these results suggest that metformin affects cardiovascular disease partly by reducing weight, but that mechanisms other than improving glycemic control or reducing insulin requirements may be of importance as well. Previous studies have shown metformin to improve endothelial function and fibrinolysis,[5, 31] independently of glycemic control, insulin requirements, or weight gain.[7] Endothelial dysfunction, in turn, has been strongly associated with an increased risk of cardiovascular disease.[13, 32-34] Metformin-associated improvements in endothelial function, however, were small.[6, 7, 31] Other possible mechanisms may include effects on advanced glycation end product levels or the secretion of adipocyte-derived mediators (such as free fatty acids, leptin, resistin, and adiponectin).[6, 35, 36] These possible mechanisms require further investigation.

Strengths of our study include its randomized, placebo-controlled, double-blind design, its long follow-up of 4.3 years, and finally the participation of patients in the trial after the occurrence of a non-fatal disease-related endpoint, thereby reducing drop-out bias. In addition, the results were consistent across the different statistical analyses used.

Our study has several limitations. First, its relatively small sample size and consequently limited power may have obscured smaller treatment effects. To increase the power of our study, disease-related endpoints were constructed by combining separate clinical events regarding micro- and macrovascular disease. An assumption in the construction of these disease-related endpoints is that its components are equally important, which is not necessarily true. In addition, the hypothesis that metformin influences both micro- and macrovascular disease through a shared underlying pathophysiology, in the way for instance obesity does, may not be correct. Diabetic foot was included in the primary endpoint, a combination of micro- and macrovascular disease, as well as in the secondary, macrovascular endpoint. The etiology of diabetic foot lesions is a complex issue, but it is recognized that three key elements are involved: neuropathy, peripheral vascular disease and infection.[37, 38] Five of the eight patients that developed a diabetic foot showed marked peripheral vascular disease on angiogram

examination during their hospital admission. However, three patients had no angiographic documentation and could have developed a diabetic foot without any peripheral arterial disease. However, the HR for the secondary macrovascular endpoint without including diabetic foot was 0.60 (0.44 to 0.85); p=0.05 compared to 0.60 (0.40 to 0.92; p=0.04). Second, there was an imbalance between the two treatment groups after randomization. We have adjusted for this in all analyses. To adjust for the difference in prior cardiovascular disease, we constructed a way of measuring cardiovascular history, which might not optimally reflect the past medical history and severity of cardiovascular disease at baseline. Therefore, the results, especially with regard to the secondary, macrovascular endpoint, must be interpreted with caution. Third, although all patients were treated in non-academic hospitals, they did receive more intensive care than normally available in such centers and our results may therefore not be generalisable to patients in other settings. Finally, we have conducted multiple analyses, and we cannot entirely exclude the possibility that the positive finding on the secondary endpoint is due to chance. However, several statistical approaches showed this to be a consistent finding.

In conclusion, we showed that in DM2 patients treated with insulin, addition of metformin resulted in improvements in weight, glycemic control, and insulin requirements, which were maintained after 4.3 years of treatment. However, metformin treatment did not reduce the risk of the primary endpoint. It may, however, reduce the risk of macrovascular disease, independently of reducing hyperinsulinemia, but partly related to the prevention of weight-gain during insulin treatment. In general practice, when, due to the progressive nature of DM2, insulin treatment is required, patients may benefit if metformin treatment is continued.

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Author Contributions

Kooy and De Jager contributed equally as primary coauthors. Kooy, De Jager, and Lehert had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept an design: Kooy, Donker, Stehouwer. Acquisition of data: De Jager, Kooy, Wulffelé, Bets. Analysis and interpretation of data: De Jager, Kooy, Lehert, Stehouwer. Drafting of the manuscript: De Jager, Kooy, Stehouwer. Critical revision of the manuscript for important intellectual content: Kooy, Donker, Stehouwer. Statistical Analysis: Lehert, De Jager, Kooy. Obtained funding: Kooy.

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Role of the Sponsors

The sponsors had no role in the design and conduct of the study; in the collection, analysis, and interpretation of the data; or in the preparation, review, or approval of the manuscript.

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Chapter 5

Metformin improves endothelial function in type 2 diabetes treated with insulin: a long-term, randomised, placebo-controlled trial

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Abstract

Background

We investigated whether metformin can improve endothelial function and decrease inflammatory activity, and thereby decrease risk of atherothrombotic disease.

Methods and results

We studied 390 patients treated with insulin in the outpatient clinics of 3 hospitals in a randomized, placebo-controlled trial with a follow-up period of 4.3 years. Either metformin 850 mg, or placebo (1-3 times daily) was added to insulin therapy. Metformin significantly reduced levels of vWF, sVCAM-1, t-PA, PAI-1, and sICAM-1, which, except for sICAM-1, were partly (about 60%) independent of metformin-associated changes in HbA_{1c}, insulin dose, and weight. No effects were found on urinary albumin excretion or CRP. The improvements in vWf and sVCAM-1 explained about 35% of the reduction in the risk of macrovascular morbidity and mortality associated with metformin treatment in this study, as published previously.

Conclusions

Metformin has specific effects on endothelial function, which may explain, in part, why metformin appears to be associated with a decreased risk of cardiovascular disease in type 2 diabetes.

Introduction

Up to 75% of patients with type 2 diabetes will die of a cardiovascular complication,[1] making prevention of cardiovascular complications in type 2 diabetes a crucial therapeutic target.

Two key features in the pathophysiology of atherothrombosis are dysfunction of the vascular endothelium and chronic, low-grade inflammation of the vascular wall.[2] Indeed, observational studies in individuals with or without type 2 diabetes have found strong associations between markers of endothelial dysfunction and chronic, low-grade inflammation on the one hand and increased risk of atherothrombotic disease on the other.[3-6]

In the context of type 2 diabetes, metformin is one of few antihyperglycaemic agents that has been associated with improvements in cardiovascular morbidity and mortality, an effect which appears to be at least in part independent of improvement in glycaemic control[7] and other conventional risk factors, such as hypertension, obesity and dyslipidaemia.[8]

These data raise the question of whether metformin can improve endothelial function and decrease inflammatory activity, and thereby decrease risk of atherothrombotic disease. There is some evidence from short-term studies that this may be the case[9-12], but no long-term, placebo-controlled data are available, and most short-term studies have focused on markers of fibrinolysis only,[10-12] which may or may not reflect endothelial function.[3]

In view of these considerations, we studied the effects of metformin treatment on endothelial function and low-grade inflammation in patients with insulin-treated type 2 diabetes in a placebo-controlled trial with 4.3 years of follow-up, the main results of which have been previously reported.[8]

Methods

Patients

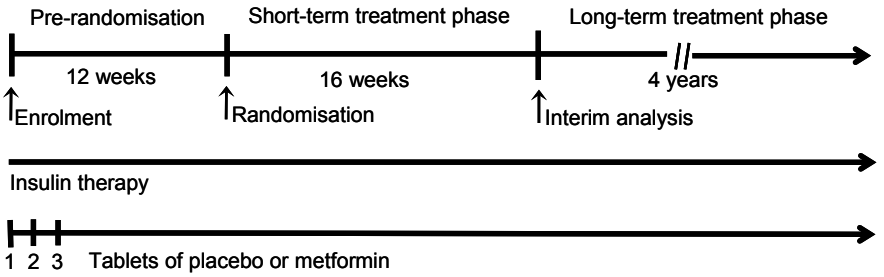
This study was part of a randomised trial investigating the effects of metformin on metabolism, micro- and macrovascular disease. We included 390 patients with type 2 diabetes (age 30-80 years) as previously described.[8, 13] All patients gave written informed consent. The medical ethical committees of the three participating hospitals approved the trial protocol. The trial has been conducted in accordance with the Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) dated 17 July 1996 and in accordance with the Declaration of Helsinki (revised version of Hong Kong in 1989 and Edinburgh in 2000).

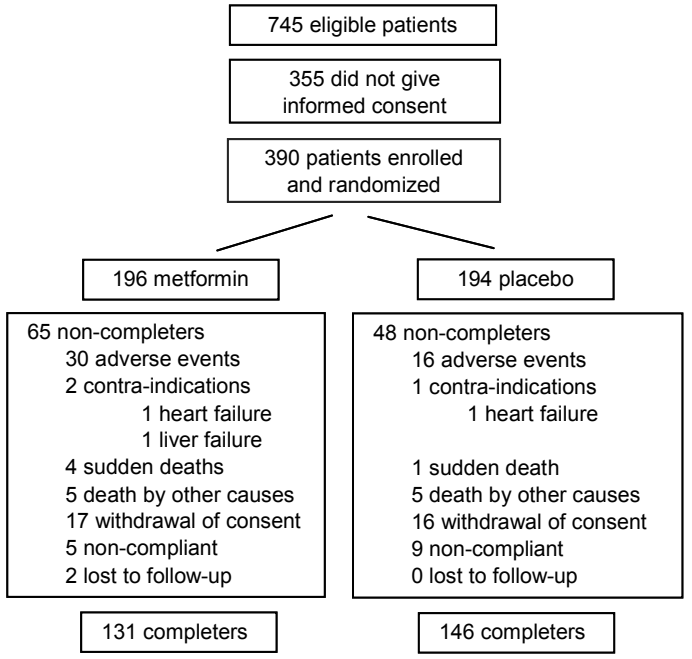
Study design (Figure 1)

The HOME trial was conducted in the outpatient clinics of three non-academic hospitals (Hoogeveen, Meppel, and Coevorden; The Netherlands). Patients were randomly

allocated to either placebo or metformin by aid of a computer program, which allocated a random number to identical looking boxes of either metformin or placebo. The trial design consisted of three phases: the 12-week pre-randomisation phase, in which patients were treated with insulin only, and concomitant medication was discontinued; the 16 week short-term treatment phase, at the beginning of which patients were randomised to receive either metformin or placebo in addition to insulin therapy; and the 48 month long-term treatment phase. After the short-term treatment phase an interim analysis took place, during which the treatment codes were not disclosed to the investigators, which was followed by the long-term treatment phase, a continuation of the short-term treatment phase.[8, 13, 14]

Figure 1. HOME trial profile and flow chart





Visits and data collection

Patients visited the clinics at the start of the pre-randomisation phase (three months before randomisation), at baseline (randomisation to metformin or placebo), one month after baseline (to check the tolerance of the drug titration), and subsequently every three months until the end of the trial. During these visits a physical examination was carried out, a medical history was taken, and laboratory and urinary investigations were performed.

Laboratory investigations

Urinary albumin excretion was measured by immunoturbidimetry (Roche Diagnostics, Basel, Switzerland) in Meppel and Hoogeveen and by means of nephelometry (BN ProSpec, Dade Behring, Marburg, Germany) in Coevorden, as described before.[15] Method comparison according to Passing and Bablok[16, 17] showed no significant deviation between these methods. Urinary albumin excretion was expressed as the albumin-to-creatinine ratio. Blood samples were drawn at baseline, after four, 17, 30, 43, and 52 months, and stored at -80°C until analysis. Plasma von Willebrand factor (vWf) antigen was measured by in-house sandwich enzyme immunoassay, using rabbit anti-vWf antigen IgG as a catching antibody and a peroxidase-conjugated rabbit anti-vWf-antigen as detecting antibody (Dako, Copenhagen, Denmark). O-Phenylenediamine (Sigma Chemical Co., St. Louis, USA) was used as substrate. Levels of vWf are expressed as percentage of antigen levels in normal pooled plasma, which is defined as 100%. The intra- and inter-assay coefficients of variation are 2.7% and 5.9%, respectively. Soluble vascular adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1) and soluble E-selectin (sE-selectin) were measured in duplicate by use of commercially available ELISA kits (Diaclone, Besançon, France). The intra- and inter-assay coefficients of variation are 1.1% and 3.1% for sVCAM-1, 1.8% and 4.2% for sICAM-1, and 2.7% and 5.9% for sE-selectin, respectively. The analysis of high sensitive C-reactive protein (CRP) was carried out on a Modular Analytics P800 system (Roche, Basel Switzerland) by using the Roche Diagnostics Tina-quant® high sensitive C-Reactive Protein reagent-kit, using anti-CRP antibodies coupled to latex microparticles to form an antigen-antibody complex. The intra- and inter-assay coefficients of variation are 0.4% and 2.7%, respectively. Tissue-type plasminogen activator (t-PA) antigen and plasminogen activator inhibitor-1 (PAI-1) antigen were measured in duplicate by use of a commercially available antigen ELISA reagent kit (Technoclone, Vienna, Austria). The intra- and inter-assay coefficients of variation are 2.6% and 7.9% for t-PA, and 3.8% and 6.8% for PAI-1, respectively.

In this trial, markers of endothelial function and inflammation have been measured previously in samples obtained at baseline and after 16 weeks of treatment.[15] To investigate the stability of the assay procedures, we compared previously obtained values with values obtained for the present investigation. Correlations between old and new measurements at baseline were 0.87 for urinary albumin excretion, 0.86 for vWf, 0.89 for sVCAM-1, 0.88 for sE-selectin, 0.92 for t-PA, 0.91 for PAI-1, 0.92 for CRP, and 0.91 for sICAM-1.

We considered urinary albumin excretion and plasma levels of vWf, sVCAM-1, sE-selectin, t-PA and PAI-1 to be markers of endothelial function,[18-20] and plasma levels of CRP and sICAM-1 to be markers of low-grade inflammation.[21, 22] However, sICAM-1 can be regarded as a marker of both endothelial function and inflammation.[22, 23] Other laboratory methods have been described elsewhere.[15]

Statistical analysis

The data presented concern all randomised patients for whom measurements were available at baseline (intention to treat sample using last observation carried forward; n= 388). All data with a skewed distribution were log-transformed before analysis. The end-point of interest was the percentage change of each variable from baseline, and the differences in these changes between the metformin and the placebo group. The differences between the metformin and placebo group were tested by means of a Student's t-test on log-transformed values. As log-values are not directly interpretable, the antilogs are reported instead. In case of log-transformed values, data are given as geometric mean (95% CI). We used multivariable linear regression analysis to investigate whether metformin-associated improvements in markers of endothelial function and markers of inflammation, if any, were independent of age, sex, smoking, the severity of prior cardiovascular disease ("CVD score" at baseline, as described previously[8]), and changes in HbA1c, insulin dose, and weight. A P-value <0.05 was considered statistically significant.

Mean standard deviation scores (z-scores) to combine the markers of endothelial dysfunction and inflammation[24] were not used because of the large variability of the z-scores, even after log transformation, as the purpose of the z-score, to decrease (intra-individual) variation and thereby increase statistical power, was not met.

Sample size calculations were based on expected differences in the occurrence of disease-related endpoints, as described previously.[8]

Results

Patients

We screened the medical files of all three participating outpatient clinics and identified 745 eligible patients (Figure 1). All were approached to enroll into the trial and 390 subjects gave written informed consent. 196 subjects were randomised to receive metformin (M) and 194 to receive placebo (P). Out of 390 included patients, 277 subjects (= 72%) completed the HOME trial (completers). Of the non-completers, 46 experienced adverse events (30 M, 16 P), three patients developed a contra-indication to metformin (one liver failure [M], two heart failure [one M, one P]), 15 encountered a fatal disease-related endpoint (nine M, six P), 47 withdrew their consent (22 M, 25 P),

and two patients were lost to follow-up (two M). Non-completers did not differ from completers with respect to duration of diabetes, prior occurrence and severity of cardiovascular disease, age, or weight. More women than men were non-completers (male:female 1:1.8 vs 1:1, p=0.011).[8]

Table 1. Baseline Characteristics (Intention to Treat Sample)

		Placebo (n=194)	Metformin (n=196)
Demography			
Men/women *n*		97/97	81/115
Age *(years)*		59 (11)	64 (10)
Currently smoking *n (%)*		59 (30)	38 (19)
Duration of diabetes *(years)*		12 (8)	14 (9)
Insulin treatment *(years)*		6 (6)	7 (8)
Concomitant medication			
Acetylsalicylic acid *n (%)*		82 (42)	78 (40)
Lipid-lowering drugs *n (%)*		31 (16)	32 (16)
Blood-pressure-lowering drugs *n (%)*		75 (39)	93 (47)
ACE inhibitors *n (%)*		31 (16)	35 (18)
Metabolic variables			
Weight *(kg)*		87 (15)	85 (16)
Body mass index *(kg/m^2)*		30 (5)	30 (5)
Waist-to-hip ratio	Men	1.03 (0.1)	1.02 (0.1)
	Women	0.93 (0.1)	0.92 (0.1)
Plasma HbA1c *(%)*		7.9 (1.2)	7.9 (1.2)
Preprandial glucose *(mmol/l)*		8.8 (1.8)	8.6 (1.8)
Postprandial glucose *(mmol/l)*		10.2 (2.0)	10.2 (2.1)
Plasma insulin *(pmol/l)*		301 (686)	248 (545)
Daily dose of insulin *(IU/day)*		64 (25)	62 (29)
Systolic blood pressure *(mmHg)*		159 (25)	160 (25)
Diastolic blood pressure *(mmHg)*		86 (11)	86 (12)
Total cholesterol *(mmol/l)*		5.5 (1.2)	5.6 (1.3)
LDL cholesterol *(mmol/l)*		3.4 (1.0)	3.6 (1.1)
Triglycerides *(mmol/l)*		1.9 (1.5)	1.7 (1.2)
HDL cholesterol *(mmol/l)*		1.3 (0.4)	1.3 (0.4)
Diabetic complications			
Cardiovascular *n (%)*		53 (29)	59 (35)
Amputation *n (%)*		3 (2)	4 (2)
Paraesthesias *n (%)*		79 (43)	83 (49)
Cardiovascular disease severity score [a]		0.92 (1.3)	1.17 (1.4)

Data are given as mean (SD) except where indicated otherwise
[a] See Kooy et al. [8]

Table 1 shows baseline characteristics of all randomised patients. Patients randomised to metformin were slightly older than patients randomised to placebo (63.6 ± 9.6 vs. 59.1 ± 11.0 years, respectively), and had a more extensive cardiovascular history (1.17 vs. 0.92, respectively), and were less often smokers. The other characteristics were comparable between the treatment groups. The actual mean dose in the metformin group was 2050 mg in the long-term treatment phase. Although the use of statins was low at baseline, in accordance with treatment guidelines at the start of the study, its use increased to 67 patients in the metformin and 54 patients in the placebo group at the final visit. There was no difference between the two groups in the use of acetylsalicylic acid, antihypertensive drugs, especially ACE inhibitors, and statins at baseline.

Markers of endothelial function (Table 2, Figure 2)

When compared with placebo, metformin treatment was associated with an increase in urinary albumin excretion of 16% (-19 to +64; p=0.422); a decrease in vWf of 11% (-16 to -6; p<0.001); a decrease in sVCAM-1 of 5% (-8 to -3; p<0.001); a decrease in sE-selectin of 2% (-6 to +3; p=0.45); a decrease in t-PA of 15% (-20 to -9; p<0.001); and a decrease in PAI-1 of 21% (-31 to -9; p=0.001).

Markers of inflammation (Table 2, Figure 2)

When compared to placebo, metformin treatment was associated with a decrease in CRP of 17% (-31 to -1; p=0.036); and a decrease in sICAM-1 of -5% (-8 to -2; p=0.004).

Additional analyses (Table 3)

Adjustment for age, sex, smoking, and severity of prior cardiovascular disease did not materially change our results, except for CRP (-28% (-94 to +39; p=0.41), after adjustment). The effect on sICAM-1 did not change materially but was no longer statistically significant (-5% (-10 to 0; p=0.06) after adjustment). Adjustment for changes in HbA1c, insulin dose, and weight, in addition to the baseline variables above, did not materially change the results, except that, after adjustment for the change in HbA1c and insulin dose, the effect of metformin on PAI-1 and sICAM-1 was no longer significant, -23% (-50 to +3; p=0.08) and +2% (-3 to +7; p=0.52), respectively. On the other hand, after adjustment for the change in HbA1c and insulin dose, the effect of metformin on sE-selectin became significant, +10% (+18 to +24; p=0.01).

After adjustment for changes in markers of endothelial dysfunction and inflammation, the hazard ratio of the macrovascular endpoint (an aggregate score of macrovascular morbidity and mortality, published previously[8]) changed from 0.61 (0.40 to 0.92; p=0.02) to 0.81 (0.59 to 1.12; p=0.21), an attenuation of 35%. This attenuation was due to adjustments for changes in vWf and sVCAM-1 (figure 3).

Table 2. Markers of endothelial function and inflammation at baseline and after 4.3 years, metformin compared to placebo

	Baseline (t_0)		Last visit (t_f)		Change (%)			P
	Placebo (P)	Metformin (M)	Placebo (P)	Metformin (M)	P t_1 vs. t_0	M t_1 vs. t_0	M vs. P	M vs. P
Markers of endothelial function								
UAE *(mg/mmol)*	1.03 (0.79-1.35)	1.00 (0.76-1.32)	0.83 (0.64-1.07)	0.84 (0.64-1.10)	-16 (-33 to +5)	-3 (-26 to +27)	+16 (-19 to +64)	0.422
vWf *(%)*	118 (112-125)	123 (117-130)	132 (125-140)	123 (116-130)	+12 (+7 to +17)	-1 (-4 to +3)	-11 (-16 to -6)	<0.001
sVCAM-1 *(ng/ml)*	744 (718-771)	766 (740-794)	779 (748-810)	762 (734-791)	+5 (+3 to +7)	-1 (-3 to +1)	-5 (-8 to -3)	<0.001
sE-selectin *(ng/ml)*	84.8 (79.2-90.7)	87.1 (82.0-92.5)	81.8 (76.3-87.7)	82.4 (77.1-88.0)	-3 (-7 to 0)	-5 (-8 to -2)	-2 (-7 to +3)	0.449
t-PA *(ng/ml)*	6.7 (6.3-7.2)	6.9 (6.5-7.4)	7.1 (6.7-7.6)	6.21 (5.8-6.7)	+6 (+1 to +11)	-10 (-14 to -6)	-15 (-20 to -9)	<0.001
PAI-1 *(ng/ml)*	45.8 (39.8-52.7)	39.7 (34.3-46.1)	48.7 (42.0-56.4)	33.6 (28.7-39.4)	+7 (-4 to +19)	-15 (-23 to -7)	-21 (-31 to -9)	0.001
Markers of inflammation								
CRP *(mg/l)*	3.06 (2.61-3.58)	3.06 (2.63-3.56)	3.12 (2.67-3.66)	2.61 (2.24-3.05)	+7 (-6 to +21)	-12 (-22 to 0)	-17 (-31 to -1)	0.036
sICAM-1 *(ng/ml)*	488 (470-507)	491 (472-511)	506 (486-527)	484 (463-506)	+4 (+1 to +6)	-1 (-4 to +1)	-5 (-8 to -2)	0.004

Data at baseline and follow-up are presented as mean with 95% CI, or, when log-transformed, as geometric mean with 95% CI. Change is expressed as the mean percentage of change accompanied with a 95% CI.

Table 3. Percentage of change from baseline, metformin as compared to placebo, 95% CI and p values

	Unadjusted		Adjusted for baseline variables		Adjusted for baseline and metabolic variables	
Markers of endothelial function						
UAE	+16 (-19 to +64)	0.42	-33 (-86 to +19)	0.22	-37 (-92 to +18)	0.18
vWf	-11 (-16 to -6)	<0.001	-13 (-23 to -6)	<0.001	-8 (-15 to -1)	0.03
sVCAM-1	-5 (-8 to -3)	<0.001	-6 (-10 to -3)	<0.001	-4 (-9 to -2)	0.03
sE-selectin	-2 (-6 to +3)	0.45	0 (-8 to +8)	0.97	+10 (+18 to +24)	0.01
t-PA	-15 (-20 to -9)	<0.001	-15 (-22 to -8)	<0.001	-9 (-16 to -2)	0.01
PAI-1	-21 (-31 to -9)	0.001	-38 (-64 to -12)	0.001	-23 (-50 to +3)	0.08
Markers of inflammation						
CRP	-17 (-31 to -1)	0.04	-28 (-94 to +39)	0.41	-5 (-76 to +66)	0.88
sICAM-1	-5 (-8 to -2)	0.004	-5 (-10 to 0)	0.06	+2 (-3 to +7)	0.52

Unadjusted results, adjusted for baseline variables (age, sex, smoking, and severity of prior cardiovascular disease), and adjusted for both baseline (age, sex, smoking, severity of prior cardiovascular disease), and metabolic variables (on-treatment changes in HbA1c, insulin dose, and weight). UAE, urinary albumin excretion; vWf, von Willebrand factor; sVCAM-1, soluble vascular cell adhesion molecule-1; t-PA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; CRP, C-reactive protein; sICAM-1, soluble intercellular adhesion molecule-1.

Figure 2. Markers of endothelial function and inflammation in time, metformin compared to placebo

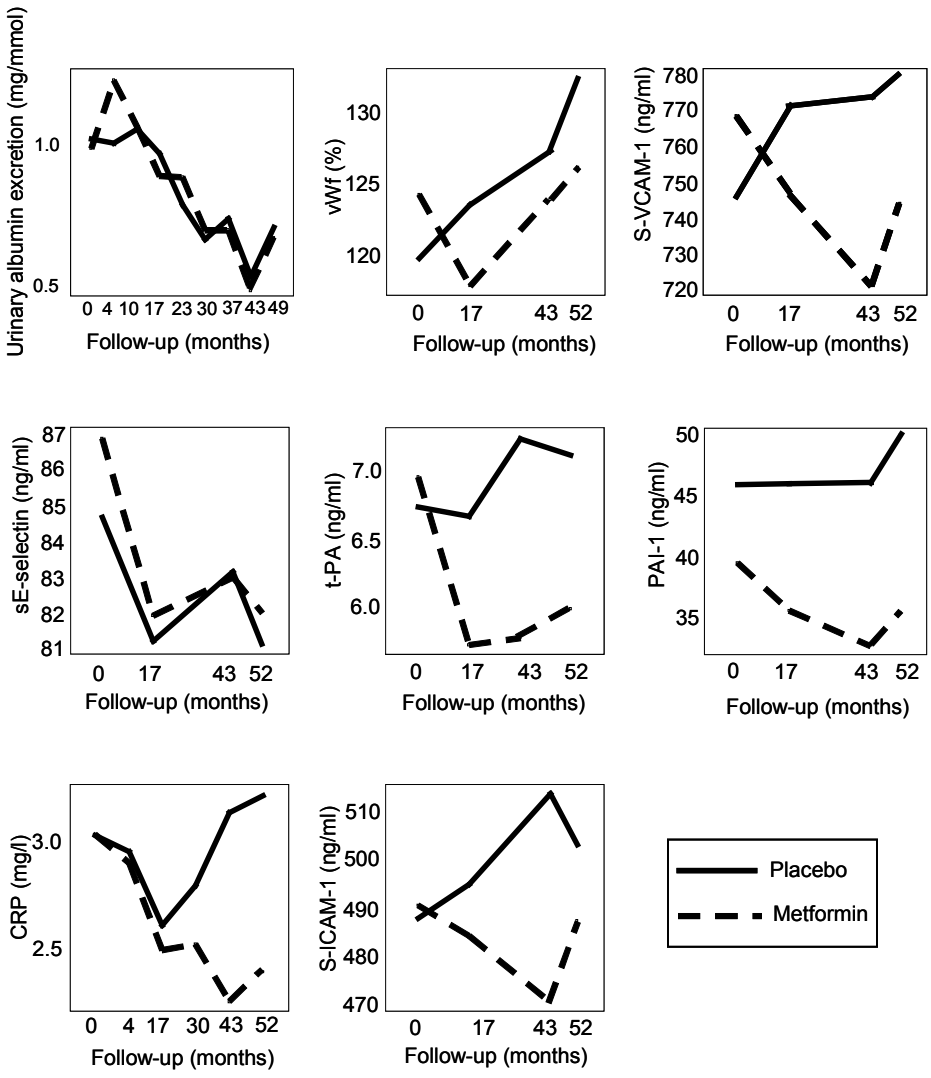
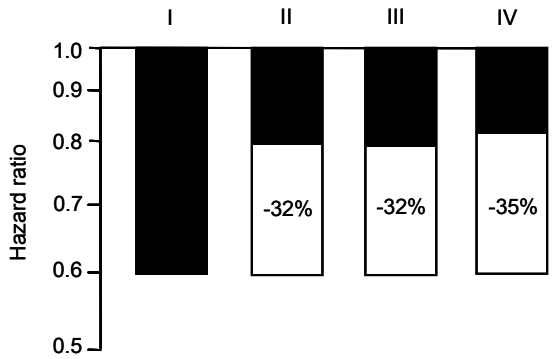


Figure 3. Hazard ratios of the macrovascular, disease-related endpoint associated with metformin treatment compared to placebo in type 2 diabetic patients treated with insulin[8]



I. Unadjusted, II. Adjusted for metformin-associated changes in sVCAM-1;
III. Adjusted for metformin-associated changes in vWf; IV. Adjusted for metformin-associated changes in both sVCAM-1 and vWf

Discussion

Our study on the long-term effects of metformin treatment on endothelial function and inflammation in patients with type 2 diabetes treated with insulin had four main findings. Firstly, metformin significantly reduced levels of vWF, sVCAM-1, t-PA, PAI-1, and sICAM-1. Importantly, our study shows that this is not a transitory phenomenon, but one that persists over time. Secondly, the favourable changes in vWF, sVCAM-1, t-PA and PAI-1 appeared to be partly independent of metformin- associated changes in glycaemic control, insulin dose, and weight, whereas the reduction in sICAM-1 was entirely mediated through metformin-induced improvements in glycaemic control and lower insulin levels. Thirdly, after adjustment for baseline imbalances in age, sex, smoking, and prior cardiovascular disease, metformin did not reduce CRP levels. Fourthly, metformin-associated changes in endothelial dysfunction, as estimated by vWF and sVCAM-1, explained about 35% of the reduction in the risk of macrovascular morbidity and mortality associated with metformin, changing the hazard ratio from 0.61 (0.40 to 0.92; p=0.02) before adjustment to 0.81 (0.59 to 1.12; p=0.21) afterwards. Taken together, these results suggest that, in type 2 diabetes, metformin decreases the risk of macrovascular disease at least in part through improving endothelial dysfunction.

Type 2 diabetes is a state of generalised endothelial dysfunction, i.e. there is impairment of many endothelial functions, such as regulation of vasomotor tone, leukocyte adhesion, haemostasis and fibrinolysis, in many vascular beds.[18] We have previously found that endothelial dysfunction in type 2 diabetes is progressive over time[24], is strongly associated with cardiovascular disease risk[5], and can explain ~34% of the excess cardiovascular mortality associated with type 2 diabetes.[24] Against this background, the current results show that metformin treatment was associated with

decreases in the plasma levels of vWf, sVCAM-1, t-PA, and PAI-1, i.e. with improvement of the endothelial regulation of haemostasis (vWf), leukocyte adhesion (sVCAM-1 and possibly sICAM-1), and fibrinolysis (t-PA and PAI-1). For vWf, t-PA and PAI-1, these findings are in accordance with previous experience in diabetic and nondiabetic individuals.[10-12, 25] To the best of our knowledge, no placebo-controlled data on the effect of metformin on sVCAM-1 in diabetic patients exist, whereas actively controlled data on sVCAM-1 in (non)diabetic patients have so far yielded contradictory results.[25-28] Interestingly and importantly, changes in the plasma levels of vWf, sVCAM-1, t-PA and PAI-1 were partly (~60 to 70%) independent of metformin-associated favourable changes in body weight, glycaemic control, and insulin dose. Taken together, these findings raise the possibility that improvement of endothelial function by metformin may represent a partly glucose-independent pathway through which metformin decreases risk of cardiovascular disease in type 2 diabetes. [7]

An important assumption in this reasoning is that plasma levels of these markers are valid indicators of endothelial function. This, in turn, requires that endothelial cells are the major source of the plasma concentrations of these proteins, and that protein concentration is determined by synthesis rather than by clearance. The validity of these assumptions is uncertain.[18] Of the markers investigated, only sE-selectin and t-PA are synthesised exclusively by endothelial cells. However, t-PA in plasma binds to PAI-1, and t-PA concentrations may mainly reflect the concentration of PAI-1, which is synthesised not only by endothelial cells, but also by hepatocytes and adipocytes. In addition, there is virtually no information on the regulation of the clearance of these proteins in type 2 diabetes, except for vWf, for which there is indirect evidence that its plasma concentration, in type 2 diabetes, is determined by synthesis rather than clearance.[29]

Urinary albumin excretion tended to increase during short-term treatment with metformin in this study, as shown previously,[15] which was then thought to be a chance finding. The current long-term results seem to confirm this explanation of the unexpected short-term findings, as no effect of metformin on urinary albumin excretion was found after 4.3 years. Previous studies of the effect of metformin on urinary albumin excretion showed either no effect or a decrease.[10, 30] The interpretation of the finding that metformin improves endothelial dysfunction, but not urinary albumin excretion, is unclear. Urinary albumin excretion depends on glomerular albumin permeation (itself dependent on pressure, permeability and surface area) and tubular reabsorption. Microalbuminuria has been strongly associated with endothelial dysfunction and is postulated to reflect increased endothelial permeability to macromolecules.[31] The simplest explanation for our findings, therefore, is that metformin did not improve endothelial permeability even if it did improve other endothelial functions.

We found no long-term effect of treatment with metformin on sE-selectin. Unexpectedly, after adjustments for changes in HbA1c and insulin dose, treatment with metformin was associated with an increase in sE-selectin levels, suggesting that metformin may have an intrinsic increasing effect on sE-selectin, which is obscured by the lowering effect of metformin-induced improvements in glycaemic control on sE-selectin. Most previous studies showed either no significant reductions in sE-selectin, or

decreases that were attributable to changes in lipid profile or glycaemic control.[26-28, 32, 33] In vitro in human endothelial cells, metformin dose-dependently inhibited TNF-α–induced NF-κB dependent gene expression of E-selectin, possibly through AMP-protein kinase activation.[34, 35] The metformin-associated increase in plasma sE-selectin levels is therefore difficult to explain and may theoretically be related to increased shedding from the cell membrane, or to decreased clearance of plasma sE-selectin. Although these possibilities require further investigation, it is important to note that we did not find any evidence that the metformin-associated increase in plasma sE-selectin affected the risk of macrovascular events.

Metformin did not reduce levels of CRP after adjusting for the baseline variables age, smoking, and prior cardiovascular disease severity. Metformin did reduce sICAM-1 levels, which was explained by metformin-associated improvements in glycaemic control and insulin levels. Studies on sICAM-1 and CRP, in diabetic and non-diabetic patients, have so far yielded contradictory results.[25, 27, 28, 36-39] One interpretation of the present data is that metformin can decrease some aspects of the low-grade inflammatory state that is common in type 2 diabetes. Alternatively, however, the decrease in sICAM-1 can be interpreted as reflecting improvement of endothelial function. Studies of other markers of inflammation, such as interleukin 6, tumour necrosis factor alpha and serum amyloid A protein, may be useful to distinguish between these possibilities.

Epidemiological data have shown endothelial dysfunction to be able to explain about 34% of the increased cardiovascular mortality associated with type 2 diabetes.[24] Metformin has been associated with lower cardiovascular morbidity and mortality.[7, 8] In our study, metformin improved endothelial dysfunction and these changes in endothelial function, specifically in vWF and sVCAM-1, explained about 30% of the reduced risk of macrovascular morbidity and mortality associated with metformin.

We showed that the effects of metformin on endothelial function were partly unrelated to decreases in hyperglycaemia, insulin dose, and weight, suggesting that metformin may have some direct effects on the endothelium.[40-42] Alternatively or additionally, metformin may improve endothelial function by decreasing advanced glycation endproduct levels,[43-45] by altering the secretion of adipocyte-derived mediators (such as free fatty acids, leptin, resistin, and adiponectin),[46-52] by decreasing inflammatory activity in ways not reflected by CRP and sICAM-1,[53, 54] and (or) by improving insulin sensitivity, of which a change in insulin dose may be an insufficiently accurate marker. These possibilities require further study.

Strengths of our study include its randomised, placebo-controlled, double-blind design; its relatively long follow-up of 4.3 years with frequent serum collection; and its non-academic setting, and therefore its value in a community setting. This study had limitations as well. Unfortunately, there was an imbalance between the two treatment groups after randomization. Although we adjusted for this in all analyses, we cannot rule out some residual confounding. In addition, the results on low-grade inflammation must be interpreted with caution. Low-grade inflammation was estimated by two markers, i.e. CRP and sICAM-1, whereas endothelial dysfunction was represented by six markers.

This may have led to an underestimation of the effect of metformin on low-grade inflammation.

We conclude that, in patients with type 2 diabetes treated with insulin, 4.3 years of metformin treatment, as compared to placebo, was associated with improvements in plasma markers of vWF, sVCAM-1, t-PA, PAI-1, and sICAM-1, which, except for sICAM-1, were partly independent of changes in HbA_{1c}, insulin dose, and weight. Metformin may thus have specific effects on endothelial function, which may explain, in part, why metformin appears to be associated with a decreased risk of cardiovascular disease in type 2 diabetes. In our study, improvements in endothelial dysfunction (i.e. vWf and sVCAM-1) explained about 35% of the reduced risk of macrovascular morbidity and mortality associated with the use of metformin.

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Disclosure
No conflict of interest exists for any of the authors.

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Chapter 6

Long-term treatment with metformin in patients with type 2 diabetes treated with insulin and risk of vitamin B12 deficiency: a randomised, placebo-controlled trial

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Abstract

Objectives

To study the effects of metformin on the incidence of vitamin B12 deficiency (vitamin B12 levels <150 pmol/l) and of low vitamin B12 levels (vitamin B12 levels below 220 but above 150 pmol/l), and on folate and homocysteine concentrations

Design

Multicenter randomised placebo-controlled trial

Setting

Outpatient clinics of three hospitals in the Netherlands

Participants

390 patients with type 2 diabetes treated with insulin

Intervention

Addition of 850 mg metformin or placebo three times daily for 4.3 years

Main outcome measures

Vitamin B12 levels, folate, and homocysteine levels measured in serum at baseline, after four, 17, 30, 43, and 52 months

Results

Metformin treatment was associated with decreases in vitamin B12 of -19% [(-24 to -14); p<0.001 vs. placebo] and folate of -5% [(-10 to -0.4); p=0.033], and an increase in homocysteine of +5% [(-1 to +11); p=0.091]. After adjustment for BMI and smoking, no significant treatment effect of metformin on folate levels was found. The risk difference at study end for vitamin B12 levels below 150 pmol/l was 7.2%-points (95% CI: 2.3 to 12.1, p=0.004); number needed to harm per 4.3 years 13.8 (95% CI: 43.5 to 8.3); the risk difference at study end for vitamin B12 levels between 150 and 220 pmo/l was 11.2%-points (95% CI: 4.6 to 17.9, p=0.001); number needed to harm 8.9 (95% CI: 21.7 to 5.6)]. Patients with vitamin B12 levels below 150 pmol/l at study end had a mean homocysteine level of 23.7 µmol/l (95% CI 18.8 to 30.0 µmol/l) compared to 18.1 µmol/l (95% CI 16.7 to 19.6 µmol/l; p= 0.003) for patients with vitamin B12 levels between 150 and 220 pmol/l and 14.9 µmol/l (95% CI 14.3 to 15.5; p<0.001 compared to B12 below 150 pmol/l; p=0.005 compared to B12 between 150 and 220 pmol/l) for patients with a vitamin B12 level above 220 pmol/l.

Conclusions

Long-term treatment with metformin causes vitamin B12 deficiency (number needed to harm per 4.3 years, 13.8), resulting in higher homocysteine levels. Because such vitamin B12 deficiency is preventable, these data suggest that regular measurement of vitamin B12 levels during long-term metformin treatment should be strongly considered.

Introduction

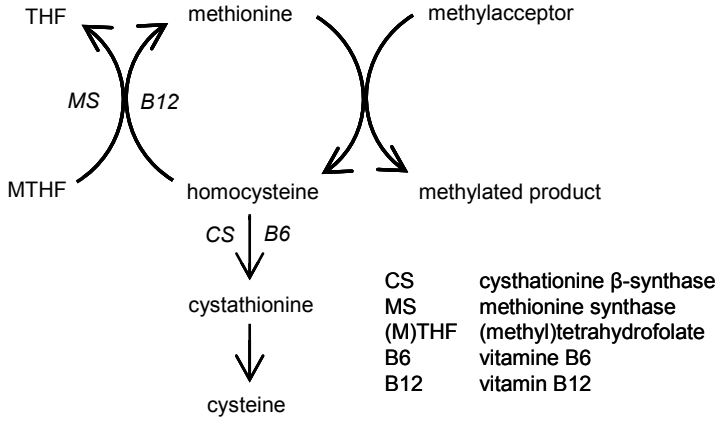
Metformin is considered one of the cornerstones in the treatment of type 2 diabetes and is the most frequently prescribed first-line therapy for patients with type 2 diabetes.[1] In addition, it is one of few antihyperglycaemic agents associated with improvements in cardiovascular morbidity and mortality,[2,3] which is the major cause of death in individuals with type 2 diabetes.[4]

There are few disadvantages to the use of metformin. However, metformin induces vitamin B12 malabsorption, which may increase the risk of developing vitamin B12 deficiency,[5-7] a clinically important and treatable condition. In addition, metformin treatment has been reported to be associated with decreased folate concentrations, although the mechanism of this effect has not been elucidated.[8] Finally, both decreases in folate and vitamin B12 levels might, in turn, result in an increase in homocysteine levels (Figure 1), an independent risk factor for cardiovascular disease, especially among individuals with type 2 diabetes.[9-11]

All current evidence on metformin-associated vitamin B12 deficiency comes from short-term studies.[5-7,12-14] No long-term, placebo-controlled data on the effects of metformin on levels of vitamin B12 in type 2 diabetes have been reported. In addition, placebo-controlled data on the effects of metformin on homocysteine in type 2 diabetes are sparse[12,15] and again no long-term data on this issue are available.

In view of these considerations, we studied the effects of metformin treatment on serum levels of vitamin B12, folate, and homocysteine in patients with type 2 diabetes in a placebo-controlled trial with 4.3 years of follow-up.

Figure 1. Homocysteine metabolism



Methods

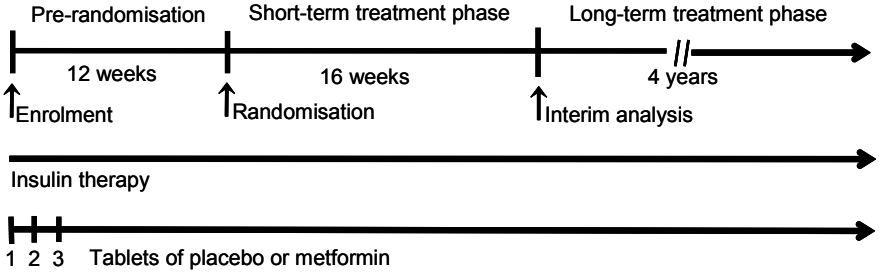
Patients

This study was part of a randomised trial investigating the effects of metformin on metabolism, micro- and macrovascular disease. We included 390 patients with type 2 diabetes (age 30-80 years) as previously described.[3,16] All patients gave written informed consent. The medical ethical committees of the three participating hospitals approved the trial protocol. The trial has been conducted in accordance with the Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) dated 17 July 1996 and in accordance with the Declaration of Helsinki (revised version of Hong Kong in 1989 and Edinburgh in 2000).

Study design (Figures 2 and 3)

The HOME trial was conducted in the outpatient clinics of three non-academic hospitals (Hoogeveen, Meppel, and Coevorden; The Netherlands). Patients were randomly allocated to either placebo or metformin by aid of a computer program, which allocated a random number to identical looking boxes of either metformin or placebo. The trial design consisted of three phases: the 12-week pre-randomisation phase, in which patients were treated with insulin only, and concomitant medication was discontinued; the 16 week short-term treatment phase, at the beginning of which patients were randomised to receive either metformin or placebo in addition to insulin therapy; and the 48 month long-term treatment phase. After the short-term treatment phase an interim analysis took place, during which the treatment codes were not disclosed to the investigators, which was followed by the long-term treatment phase, a continuation of the short-term treatment phase.[3,12,16]

Figure 2. HOME trial profile

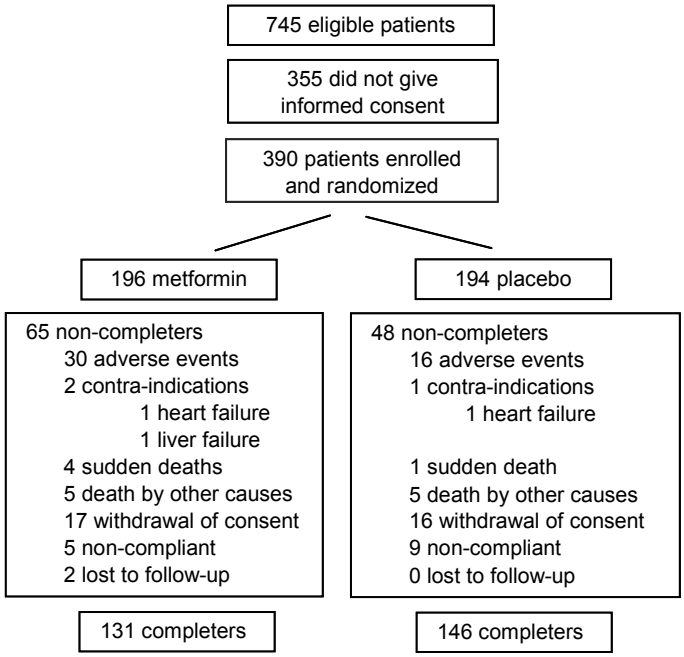


Visits and data collection

Patients visited the clinics at the start of the pre-randomisation phase (three months before randomisation), at baseline (randomisation to metformin or placebo), one month after baseline (to check the tolerance of the drug titration), and subsequently every three

months until the end of the trial. During these visits a physical examination was carried out, a medical history was taken, and laboratory investigations were performed.

Figure 3. HOME trial flow chart



Laboratory investigations

Blood samples for this study were drawn at baseline, after four, 17, 30, 43, and 52 months, and stored at -80°C until analysis. Vitamin B12, folate, and homocysteine were measured in serum. Vitamin B12 and folate concentrations are determined by an electrochemiluminescence immunoassay (ECLIA) using the competition principle. The mean intra-assay coefficients of variation for vitamin B12 and folate were 2.3% and 3.5%, respectively. The mean inter-assay coefficients were 2.9% and 4.7%, respectively.

Total homocysteine concentration was measured by using a kit for homocysteine from Chromsystems (Martinsried, Germany). The results were corrected against two levels of 'consensus plasma samples' (SKML, Nijmegen, the Netherlands) with concentrations of 13 and 55 micromoles per liter. The correction factor found was 0.90. The intra-assay coefficients of variation were 2.2% and 1.8% at the level of 12.8 and 72.2 micromoles per litre, respectively. The inter-assay coefficients of variation were 6.1% and 5.2% at the level of 9.8 and 21.1 micromoles per litre, respectively.

In this trial, vitamin B12, folate, and homocysteine levels have been measured previously in samples obtained at baseline and after 16 weeks of treatment.[12] To investigate the stability of the assay procedures, we compared previously obtained[12] values with values obtained for the present investigation. Correlations between old and new measurements at baseline and after 16 weeks were 0.58 and 0.91, respectively for vitamin B12, 0.90 and 0.83 for folate, and 0.99 and 0.99 for homocysteine. The relatively low correlation for vitamin B12 values at baseline was caused by 5 cases for which a large discrepancy existed between old and new values, which were subsequently excluded from analyses involving vitamin B12.

Statistical analysis

We log-transformed data on vitamin B12, folate, and homocysteine before analysis, because of their skewed distribution. Data are given as geometric mean (95% confidence intervals). As log values are not directly interpretable, the antilogs are reported instead. These values are the geometric mean percentages of change from baseline. The end-point of interest was the percentage change of each variable from baseline, which was calculated from baseline values and the summary mean. The differences between the metformin and the placebo group were tested by a central t-test on log-transformed values. We also calculated the hazard ratio for developing vitamin B12 deficiency, which was defined as vitamin B12 levels below the value of 150 pmol/l, and low vitamin B12 levels, which was defined as vitamin B12 levels below 220 but above 150 pmol/l.[17] All analyses were based on intention-to-treat samples using the last-observation-carried-forward procedure. To test whether results obtained were robust, we also used Mixed Models analysis to impute missing data. Patients with vitamin B12 levels below 150 pmol/l at baseline and/or at the interim analysis at 16 weeks (n=8) were supplemented and therefore excluded from analyses after 16 weeks.

We used Linear Mixed Models to explore the effects of metformin on vitamin B12, folate and homocysteine, and to investigate whether metformin-associated changes in homocysteine, if any, could be explained by changes in folate and (or) vitamin B12, and, if so, whether the changes were independent of age, gender, duration of diabetes, smoking, body mass index, insulin dose, serum creatinine, HDL cholesterol, or glycated haemoglobin. The goodness of fit between alternative models was compared using the maximum likelihood technique.

Sample size calculations were based on expected differences in the occurrence of disease-related endpoints, as described previously.[3] However, with the current sample size, a decrease in vitamin B12 levels of 5% in the metformin group compared to placebo by using ANCOVA tests should be detectable at a two-sided, 95% confidence level, with a power of 0.82.

P-values <0.05 were considered statistically significant.

Results

Patients (Table 1, Figures 2 and 3)

We screened the medical files of all three participating outpatient clinics and identified 745 eligible patients. All were approached to enroll into the trial and 390 subjects gave written informed consent. 196 subjects were randomised to receive metformin and 194 to receive placebo. Out of 390 included patients, 277 subjects (= 72%) still received metformin or placebo at the end of the trial. Figure 3 shows the flow of patients through the trial. 46 patients (30 metformin, 16 placebo) discontinued because of adverse effects, which have been described more extensively elsewhere.[3] Only 2 participants were lost to follow-up (at 33 and 26 months, respectively).

Tabel 1. Baseline characteristics

		Placebo (n=191)	Metformin (n=194)
Demography			
Men/women *n*		95/96	81/113
Age *(years)*		59 (11)	64 (10)
Currently smoking *n (%)*		59 (30)	38 (19)
Duration of diabetes *(years)*		12 (8)	14 (9)
Insulin treatment *(years)*		6 (6)	7 (8)
Concomitant medication			
Lipid-lowering drugs *n (%)*		29 (15)	32 (17)
Blood-pressure-lowering drugs *n (%)*		74 (39)	91 (47)
Metabolic variables			
Weight *(kg)*		87 (15)	85 (16)
Body mass index *(kg/m^2)*		30 (5)	30 (5)
Waist-to-hip ratio	Men	1.02 (0.1)	1.02 (0.1)
	Women	0.92 (0.1)	0.93 (0.1)
Plasma HbA1c *(%)*		7.9 (1.2)	7.9 (1.2)
Daily dose of insulin *(IU/day)*		64 (25)	62 (29)
Systolic blood pressure *(mmHg)*		160 (25)	160 (25)
Diastolic blood pressure *(mmHg)*		86 (11)	86 (12)
Total cholesterol *(mmol/l)*		5.5 (1.2)	5.6 (1.3)
LDL cholesterol *(mmol/l)*		3.4 (1.0)	3.6 (1.1)
Triglycerides *(mmol/l)*		1.9 (1.5)	1.7 (1.2)
HDL cholesterol *(mmol/l)*		1.3 (0.4)	1.3 (0.4)
Vitamin B12 *(pmol/l)*		380 (135)	378 (130)
Folate *(nmol/l)*		18.7 (7.5)	18.7 (7.2)
Homocysteine *(µmol/l)*		14.6 (10.3)	14.4 (9.7)
Prior macro- and microvascular disease			
Myocardial infarction *n (%)*		21 (11)	24 (12)
Cardiovascular intervention *n (%)*		17 (9)	27 (14)
Stroke *n (%)*		8 (4)	8 (4)
Non-traumatic amputation *n (%)*		3 (2)	5 (3)

Data are given as mean (SD) except where indicated otherwise

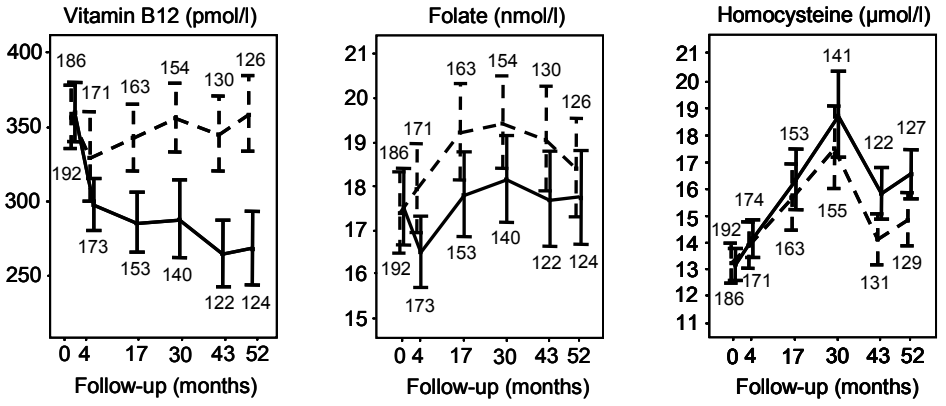
The actual mean dose in the metformin group was 2050 mg per day. At the final visit, laboratory samples were available for 256 patients (127 metformin, 129 placebo). The main outcomes of this trial have been reported previously.[3]

Table 1 shows baseline characteristics of all randomised patients. Patients randomised to metformin were older than patients randomised to placebo (64 ± 10 vs. 59 ± 11 years, respectively), had a more extensive cardiovascular history, and were less often smokers. The other characteristics were comparable between the two treatment groups.

Vitamin B12, folate, and homocysteine (Figures 4 and 5)

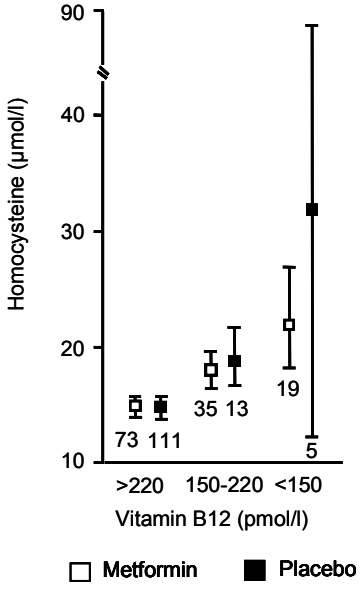
During placebo treatment, vitamin B12 increased by 0.2 pmol/l [0% (-3 to 4)], folate increased by 1.01 nmol/l [8% (4 to 12)], and homocysteine increased by 1.60 µmol/l [20% (95% CI: 16 to 25)]. During metformin treatment, vitamin B12 decreased by 89.8 pmol/l [-19% (-22 to -15)], folate increased by 0.21 nmol/l [3% (-1 to 6)], and homocysteine increased by 3.26 µmol/l [26% (21 to 31). Compared with placebo, metformin treatment was associated with decreases in vitamin B12 of 19% [(-24% to -14); p<0.001] and folate of 5% [(-10 to -0.4); p=0.033], and an increase in homocysteine of 5% [(-1 to 11); p=0.091]. The effects of metformin on vitamin B12, folate, and homocysteine were re-analysed with adjustment for age, previous metformin treatment, duration of diabetes, gender, insulin dose, and smoking habits, all of which did not materially change the results (data not shown), except for folate. After adjustment for body mass index and smoking, no significant treatment effect of metformin on folate levels was found (metformin compared to placebo, -0.1%, p-value 0.57).

Figure 4. Levels of vitamin B12, folate, and homocysteine with 95% confidence intervals. Solid lines represent the metformin group, dotted lines the placebo group. Number of available samples for the metformin and placebo group are indicated



At baseline, 3 patients (1.6%) in the metformin group and 4 (2.2%) in the placebo group had vitamin B12 levels below 150 pmol/l, while 14 patients (7.3%) and 14 patients (7.5%) had vitamin B12 levels between 150 and 220 pmol/l, respectively. At the end of the study period, 19 patients (9.9%) in the metformin group and 5 (2.7%) in the placebo group had vitamin B12 levels below 150 pmol/l, while 35 (18.2%) and 13 (7.0%) had vitamin B12 levels between 150 and 220 pmol/l, respectively [risk difference at study end for vitamin B12 levels below 150 pmol/l, 7.2%-points (95% CI: 2.3 to 12.1, p=0.004); number needed to harm 13.8 per 4.3 years (95% CI: 43.5 to 8.3); risk difference at study end for vitamin B12 levels between 150 and 220 pmol/l, 11.2%-points (95% CI: 4.6 to 17.9 , p=0.001); number needed to harm per 4.3 years 8.9 (95% CI: 21.7 to 5.6)]. The hazard ratio for developing vitamin B12 levels below 150 pmol/l when treated with metformin was 5.5 (95% CI: 1.6 to 19.1), p=0.01; for vitamin B12 levels between 150 and 220 pmol/l, 3.0 (95% CI: 1.3 to 6.6), p=0.007.

Figure 5. Homocysteine levels with 95% confidence intervals for normal vitamin B12 levels, intermediate B12 levels, and vitamin B12 deficiency after 4.3 years. Number of patients in each treatment group is indicated below each of the confidence intervals



Patients with vitamin B12 levels below 150 pmol/l at the study end had a mean homocysteine level at the study end of 23.7 µmol/l (95% CI 18.8 to 30.0 µmol/l) compared to 18.1 µmol/l (95% CI 16.7 to 19.6 µmol/l; p= 0.003) for patients with vitamin B12 levels between 150 and 220 pmol/l and 14.9 µmol/l (95% CI 14.3 to 15.5; p<0.001 compared to patients with vitamin B12 below 150 pmol/l; p=0.005 compared to patients

with vitamin B12 between 150 and 220 pmol/l) for patients with a vitamin B12 level above 220 pmol/l. Homocysteine levels did not differ significantly between treatment groups when stratified for end-of-treatment vitamin B12 level.

Linear Mixed Model

The interaction between treatment and time was a significant determinant of vitamin B12 levels (p=0.023), i.e. the vitamin B12-lowering effect of metformin increased with time. Body mass index and smoking were strong inverse determinants of folate (p-values 0.003 and <0.0001, respectively). After adjustment for body mass index and smoking, treatment with metformin was not a significant determinant of folate levels, nor was the interaction between treatment and time (p=0.57 and 0.23, respectively). Vitamin B12 and folate were strong determinants of homocysteine (p<0.0001). Homocysteine increased with age (p<0.0001). There was no significant interaction between treatment and time for homocysteine levels (p=0.16).

Additional analyses

Per protocol analysis using only available data for those patients who remained in the trial until the final visit (n=256) yielded similar results (data not shown). General mixed model analysis yielded similar results to analysis using last observation carried forward (data not shown). Because last observation carried forward is considered the more conservative analysis of the two, “freezing” any observed divergence between two groups by retaining the last observation made, compared to mixed model analysis, in which in case of missing data, estimations of future observations are made based on observations made in the past, thereby reflecting a divergence more accurately - but less conservatively - last observation carried forward was primarily used here.

Discussion

Our study on the long-term effects of metformin treatment on vitamin B12, folate, and homocysteine in patients with type 2 diabetes treated with insulin had three main findings. First, metformin significantly reduced levels of vitamin B12 (-19% [p<0.001]), in accordance with previous studies.[13,18,19] Importantly, our study shows that this is not a transitory phenomenon, but persists and increases over time. After 4.3 years of treatment, patients treated with metformin had a ~7%-point greater risk of having developed vitamin B12 deficiency than patients treated with placebo. Second, when compared to placebo, a small, significant decrease in folate levels in the metformin group was found, which however was not statistically significant after adjustments for body mass index and smoking. Third, the decrease in vitamin B12 levels was associated with an increase in homocysteine levels which was not statistically significant. Further analyses, however, showed that homocysteine levels did increase in individuals in whom vitamin B12 levels decreased below the level that is often considered to indicate clinical deficiency (i.e., 150 pmol/L).

Decreases of vitamin B12 during metformin treatment are not novel and have been reported before. However, a novel finding is that the B12 decrease is progressive, and that some patients get to levels that most authorities agree require substitution. This is also a novel finding, because earlier trials were in well-fed, middle-aged patients in whom, although metformin decreased B12 levels, these levels remained within the normal range.[5 6 14] Metformin is thought to induce malabsorption of vitamin B12 and intrinsic factor in the ileum, an effect that can be reversed by increased calcium intake.[6 18] The consequences of clinically important decreases in vitamin B12 levels such as macrocytic anaemia, neuropathy, and mental changes can be profound. We note that there is no consensus on the issue of whether "asymptomatic" B12 deficiency should be treated (The British Committee for Standards in Haematology (BCSH) Guidelines), although studies show that some symptoms of B12 deficiency are difficult to diagnose, can be irreversible, and diagnosis and treatment of vitamin B12 deficiency is relatively easy, cheap, safe and effective,[20-23] in effect arguing in favour of treatment. In addition, while the necessity of the treatment of "spontaneous" B12 deficiency may be debated, we would argue that there is an important difference between *spontaneous* and *drug-induced* B12 deficiency, as a key principle of drug prescription is to do no harm. Our study shows that it is reasonable to assume that harm will eventually occur in some patients with metformin-induced low vitamin B12 levels.

Folate increased in both the metformin and placebo groups, possibly due to dietary counselling received by all patients throughout the trial. Our short-term results showed a significantly larger increase in the placebo group,[12] a finding which was initially replicated in the current analysis, but disappeared after adjustment for body mass index and smoking. Previous studies have shown either no or small effects of metformin treatment on homocysteine.[13 14 24 25] However, we clearly show that homocysteine levels do increase with decreasing levels of vitamin B12 (Figure 5). The finding that metformin treatment significantly lowered vitamin B12 but not homocysteine levels probably reflects the relatively low incidence of vitamin B12 deficiency in the entire study population. However, it is to be expected that as treatment with metformin continues, vitamin B12 levels will continue to decrease, making increases in homocysteine levels inevitable in time.

Strengths of our study include its randomised, placebo-controlled, double-blind design; its relatively long follow-up of 4.3 years with frequent serum collection; and its non-academic setting, and therefore its value in a community setting. Limitations of our study include the fact that we measured only total vitamin B12 levels, and did not measure holotranscobalamine-II or methylmalonic acid, which may have been more precise indicators of vitamin B12 status. Finally, it is likely that, if anything, we underestimated the impact of metformin treatment on the risk of clinically important vitamin B12 deficiency, as we show that metformin treatment was associated not only with a greater risk of developing vitamin B12 levels below 150 pmol/l (odds ratio, 5.5) but also with a greater risk of developing vitamin B12 levels between 150 and 220 pmol/l (odds ratio, 3.0), which is likely to represent clinically important vitamin B12 deficiency in at least some individuals.[26] A further reason that we may have somewhat underestimated the adverse effects of metformin is that all participants in our trial

received frequent dietary counselling, which may have attenuated the impact of metformin treatment on vitamin status and which may not be available in routine clinical practice.

In conclusion, we showed that in type 2 diabetic patients treated with insulin, addition of metformin resulted in a 7%-point absolute greater risk of vitamin B12 deficiency during 4.3 years of follow-up. In addition, the metformin-associated reduction of vitamin B12 levels increased with time. Current guidelines indicate that metformin is a cornerstone of the treatment of type 2 diabetes, but make no recommendations on the detection and prevention of vitamin B12 deficiency during metformin treatment. Our data provide a strong case for routine assessment of vitamin B12 levels during long-term treatment with metformin.

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Chapter 7

Summary and general discussion

Historical perspective of the HOME trial

In 1998 the first results of the UKPDS,[1] an important landmark study on the influence of glycaemic control on diabetic complications, suggested a cardioprotective role for metformin, which seemed independent of glycaemic control. However, the design and analyses of the UKPDS raised considerable debate. In addition, the mechanisms through which metformin might influence cardiovascular disease were not fully elucidated. These questions inspired the design of the randomised, placebo-controlled, multicenter trial "Hyperinsulinaemia: the Outcome of its Metabolic Effects" (HOME) to investigate these issues.

In the late '90s when the study protocol for the HOME trial was written, it was designed to study whether metformin could lower the incidence of micro- and macrovascular disease in type 2 diabetes and, if so, if a reduction in hyperinsulinaemia could explain this improvement in micro- and macrovascular disease. Being an "insulin-sensitizer" with a long history of safe clinical use, metformin was used versus placebo, in addition to insulin treatment, to reduce hyperinsulinaemia with the aim of comparable glycaemic control between the two treatment groups. More recently, attention was first given to other conventional and later to non-conventional risk factors instead of hyperinsulinaemia as possible candidates to explain the advantageous effects of metformin (if any).

There are few disadvantages to the use of metformin. However, metformin induces vitamin B12 malabsorption, which may increase the risk of developing vitamin B12 deficiency,[2-4] a clinically important and treatable condition. In addition, metformin treatment has been reported to be associated with decreased folate concentrations, although the mechanism of this effect has not been elucidated.[5] Finally, both decreases in folate and vitamin B12 levels might, in turn, result in an increase in homocysteine levels, an independent risk factor for cardiovascular disease, especially among individuals with type 2 diabetes.[6-8]

Present findings

The association of endothelial dysfunction and inflammation and cardiovascular disease (Hoorn study)

Endothelial dysfunction and low-grade inflammation were associated with higher risks of cardiovascular mortality. For endothelial dysfunction, these associations were stronger in diabetic than in nondiabetic individuals. Both endothelial dysfunction and low-grade inflammation were associated with cardiovascular mortality independently of other cardiovascular risk factors, and also appeared mutually independent, indicating that they may represent largely distinct pathways of disease and therefore distinct targets for intervention. Low grade inflammation explained about 25%, endothelial dysfunction about 34%, and together explained about 43% of the increased mortality risks associated with type 2 diabetes.

Short-term effects of metformin on endothelial dysfunction and inflammation (HOME study)

Short-term treatment (16 weeks) with metformin was associated with decreases in the plasma levels of vWf, sVCAM-1, sE-selectin, t-PA and PAI-1. Changes in the plasma levels of these markers were independent of metformin-associated favourable changes in body weight, glycaemic control, insulin dose, and lipid profile. The only exception was the decrease in plasma sE-selectin, which was in part explained by the metformin-associated improvement in glycaemic control and triglyceride levels. We found no effect of short-term treatment with metformin on markers of low-grade inflammation, i.e. sICAM-1 and CRP.

Long-term effects of metformin on metabolism and micro- and macrovascular disease (HOME study)

Long-term treatment (4.3 years) with metformin improved body weight and insulin requirements and had moderately beneficial effects on glycaemic control (despite the aim of similar glycaemic control in both groups), but not on blood pressure and the plasma lipid profile. Metformin treatment did not decrease the risk of the primary end point, which consisted of both microvascular and macrovascular disease. However, it did decrease the risk of the secondary, macrovascular end point (hazard ratio 0.61 (95% CI, 0.40-0.94; P=0.02), which was partly (about 40%) related to the prevention of weight gain during metformin treatment. All other metformin-associated changes in metabolic or haemodynamic variables, including insulin levels, did not seem to contribute to the favourable effect of metformin on macrovascular disease. No improvement in microvascular disease was found.

Long-term effects of metformin on endothelial dysfunction and inflammation and the association with cardiovascular disease (HOME study)

Long-term treatment (4.3 years) with metformin reduced levels of vWf, sVCAM-1, t-PA, PAI-1, and sICAM-1, which, except for sICAM-1, were partly (about 60%) independent of metformin-associated changes in HbA_{1c}, insulin dose, and weight. The reduction in sICAM-1 levels was entirely mediated through metformin-induced improvements in glycaemic control and lower insulin levels. No effects were found on urinary albumin excretion or CRP. Of all markers, only vWf and sVCAM-1 were predictors of macrovascular disease; improvements in vWf and sVCAM-1 could statistically explain 35% of the reduction in the risk of macrovascular morbidity and mortality associated with metformin treatment.

Long-term effects of metformin on vitamin B12, folate, and homocysteine levels (HOME study)

Long-term treatment (4.3 years) with metformin reduced levels of vitamin B12; the hazard ratio for developing vitamin B12 levels below 150 pmol/l when treated with metformin compared to placebo was 5.5 (95% CI: 1.6 to 19.1). A small, significant decrease in folate levels in the metformin group was found, which was not statistically

significant after adjustments for differences in BMI and smoking. The decrease in vitamin B12 levels was associated with an increase in homocysteine levels, which was not significantly different between the two treatment groups. Irrespective of treatment, homocysteine levels did significantly increase in individuals in whom vitamin B12 levels decreased below the level conventionally considered to indicate clinical deficiency (i.e., 150 pmol/L).

Do other drugs used in the treatment of diabetes reduce the risk of cardiovascular disease and if so, do endothelial function and inflammation play a role?

Sulfonylurea derivatives

Sulfonylureas are widely prescribed for the treatment of type 2 diabetes. Differences in chemical structure, pharmacokinetic, and pharmacodynamic properties between sulfonylureas may lead to differences in therapeutic effects and side effects. Studies on the effects of sulfonylureas on endothelial function and inflammation have focused mainly on PAI-1. The effect seems at least partly dependent on the sulfonylurea used: some studies report improvements in levels of PAI-1 for most sulfonylureas,[9-11] but not for all.[12]

In 1975, the University Group Diabetes Program (UGDP) trial noted excess cardiac deaths in patients treated with tolbutamide.[13] Whether this was attributable to higher baseline cardiac risk in the patients allocated to tolbutamide or to a true biological effect has been widely debated. Later, several studies and meta-analyses showed no detrimental effect of sulfonylureas, or glyburide specifically, on cardiovascular disease or mortality as compared with insulin.[14, 15] However, no benefit was shown either (hazard ratios, glyburide vs. insulin for cardiovascular events 0.92 (0.71 to 1.19) vs. 0.89 (0.70 to 1.14), respectively; and death 0.79 (0.47 to 1.32) vs. 0.97 (0.79 to 1.20), respectively.

In short, no evident beneficial (or detrimental) effect of sulfonylureas on cardiovascular events or mortality has been shown, and the effects on endothelial function seem very modest at best.

Acarbose

Acarbose is an inhibitor of alpha glucosidase, an enteric enzyme that releases glucose from larger carbohydrates, thereby reducing the rate of digestion of complex carbohydrates and consequently lowering postprandial glucose levels. Only sparse evidence on the effect of acarbose on markers on endothelial function and inflammation exists. One study showed no effect of acarbose alone on levels on PAI-1, whereas acarbose in combination with a strict exercise programme did lower levels of PAI-1.[16] One study showed no effect of acarbose on forearm flow-mediated vasodilatation (FMD), while another did show an improvement in carotid intima-media thickness.[17]

However, acarbose consistently improves postprandial glucose, which has been recognised in epidemiological studies as a risk factor for cardiovascular disease,[18] which may either directly influence cardiovascular disease or indirectly by improving other cardiovascular risk factors such as body weight, hypertension and dyslipidaemia.[19-22] In one study, the beneficial effect on cardiovascular disease remained after adjusting for the effects of acarbose on triglycerides, body weight, and systolic blood pressure.[20]

There is evidence from clinical trials and meta-analyses that acarbose improves cardiovascular outcome, such as myocardial infarction.[19, 20] However, trials on mortality are sparse, relatively short-term, and underpowered [23, 24] and unambiguous conclusions on the effects on mortality cannot be drawn.

In short, acarbose improves cardiovascular outcome, and probably mortality as well, although evidence on this latter subject is weak. The evidence on the effect of acarbose on endothelial function or inflammation is very sparse. However, it shows that acarbose ameliorates some cardiovascular risk factors, such as weight gain, high blood pressure, and dyslipidaemia. In addition, there are some indications that acarbose might have effects on cardiovascular disease mediated by improving non-traditional cardiovascular risk factors, suggesting pleiotropy. The direct effect of postprandial glucose on cardiovascular disease may play a role, as well as effects on vascular endothelium and inflammation, reflected in other ways than those studied in the few trials so far.

Thiazolidinediones

Thiazolidinediones (TZDs) were introduced in the late 1990s and act by binding to PPARs (peroxisome proliferator-activated receptors), a group of receptor molecules inside the cell nucleus, specifically PPARγ (gamma), resulting in the transcription and translation of a variety of proteins involved in glucose metabolism. There is little and contradictory evidence from placebo-controlled trials on the effects of TZDs on endothelial dysfunction and inflammation. Some studies found a reduction in CRP levels,[25] an improvement in the albumin-to-creatinine ratio,[26] or a reduction in PAI-1 levels,[27] but others found no effect on levels of CRP,[28] sVCAM-1, sICAM-1, or vWf.[27,29]

More importantly, most (meta-)analyses of large intervention trials such as PROactive, ADOPT, and RECORD showed no benefit of TZDs in cardiovascular morbidity or mortality.[30-34] On the contrary, in one meta-analysis TZDs were associated with a increased risk of heart failure and myocardial infarction (hazard ratios of 2.17 (1.49 to 3.17) and 1.29 (1.01 to 1.66), respectively), without an increased risk of death.[35]

In short, it seems that TZDs have no evident effect on endothelial function and inflammation, and no beneficial effect on cardiovascular morbidity or mortality.

GLP-1 receptor agonists

More recently, a new class of antiglycaemic agent, the glucagon-like peptide 1 (GLP-1) receptor agonists were approved for the treatment of type 2 diabetes in the United States. GLP-1 receptor agonists mimic the effects of incretins, a group of gastrointestinal hormones that are released from the gastrointestinal tract in response to

nutrient ingestion. GLP-1 receptor agonists enhance the glucose-sensing and insulin secretory capacity of the endocrine pancreas during postprandial hyperglycaemia, suppress glucagon secretion and hepatic glucose output, delay gastric emptying, reduce food intake, and promote glucose disposal in peripheral tissues.[36] Recent research shows that exenatide, a synthetic GLP-1 analogue, improves β-cell function during 1 year of treatment compared with titrated insulin glargine,[37] thereby possibly preventing the ongoing deterioration of glycaemic control as shown in long-term trials that has been attributed to a progressive loss of β-cell function.[1, 38] In addition, exenatide was associated with considerable weight loss (4,6 kg) compared to insulin.[37]

No in vivo data on the effect of GLP-1 agonists on endothelial dysfunction or inflammation have yet been published. However, an in vitro experiment showed GLP-1 agonists to reduce mRNA and protein expression of PAI-1, ICAM-1, and VCAM-1.[39] One in vitro study showed no protective effect of GLP-1 on triglyceride-induced reduction of vasorelaxation in rat conduit arteries,[40] while another suggested it relaxes rat conduit arteries in an endothelium-independent manner.[41] No data on cardiovascular outcome or mortality have been published.

In short, GLP-1 agonist may prove to be very effective in the treatment of type 2 diabetes, but their effect on endothelial function, inflammation, and cardiovascular outcome has yet to be established.

ACE inhibitors and angiotensin II receptor blockers (ARBs)

ACE inhibitors and ARBs are a group of pharmaceuticals that are used primarily in treatment of hypertension and congestive heart failure. It has been well-established that both drugs reduce the development and progression of micro-albuminuria,[42] although some debate still exists whether this is a drug-specific or a antihypertensive-specific effect.[43]

One study found a reduction in CRP levels when treated with an ACE inhibitor compared to placebo on top of conventional antihypertensive therapy, but showed no effect on levels of sICAM-1, sVCAM-1, or sE-selectin [44] as did other studies.[45, 46] No effect on CRP was found in other studies.[43, 45-47] No effect was found on vWf,[43, 45] or similar reductions in sICAM-1 and sVCAM-1 were found when other antihypertensive agents were used.[43] One study found no effect on forearm flow-mediated vasodilatation (FMD),[48] another found similar effects on FMD when the same blood pressure reduction was achieved with another antihypertensive drug.[49] An endothelium-independent vasodilatory effect was shown to be responsible for an improvement in forearm blood flow in another study.[46] However, several studies consistently show both ACE inhibitors and ARBs to reduce levels of PAI-1.[50-53]

Meta-analyses showed a reduction in cardiovascular morbidity and all-cause mortality in diabetic patients when treated with ACE inhibitors,[54, 55] but not ARBs.[55] Again, other studies showed improvements in cardiovascular morbidity and mortality when reducing blood pressure, irrespective of the drug used.[56]

There is convincing evidence that ACE inhibitors reduce cardiovascular morbidity and all-cause mortality. It remains unclear whether this is largely due to the reduction of blood pressure, or if other intrinsic effects of ACE inhibitors may influence

cardiovascular disease. Evidence indicates that ACE inhibitors do have an effect on PAI-1, but probably not on other markers of endothelial dysfunction or inflammation. ACE inhibitors may improve endothelial function and inflammation in other ways than reflected by plasma markers, such as preventing angiotensin-II-induced vasoconstriction, stimulating the release of NO and other vasodilator substances as a reaction to increased levels of bradykinin, or by ameliorating oxidative stress.[57]

In short, ACE inhibitors convincingly reduce cardiovascular disease and mortality, but probably not by improving endothelial function in the way metformin does, i.e. represented by improvements in plasma markers. They may however, beneficially influence the vascular endothelium in other ways.

Other antihypertensive drugs

Except for studies with ACE inhibitors and ARBs, not many studies on the effect of antihypertensive agents on endothelial function and inflammation exist. One study showed an improvement in FMD with β-blockers, but no effect on CRP.[58] In addition to ACE inhibitors, calcium channel blockers have been shown to improve fibrinolysis.[59] The effect on fibrinolysis was partly independent of the blood pressure reducing effect of both drugs.[59] One trial, studying the effect of hydrochlorothiazide, ACE inhibitor, and ARB in a randomised setting, showed similar improvements in urinary albumin excretion (UAE), sVCAM-1, and sICAM-1 for all agents, but no effect on CRP and vWf.[43] The improvements in UAE, sVCAM-1, and sICAM-1 were largely dependent on the blood pressure lowering effect in the hydrochlorothiazide group, but not so in de ACE inhibitor or ARB group.[43] Several studies on antihypertensive agents and their effect on carotid intima media thickness have been done. Meta-analyses show several antihypertensive drugs to reduce carotid intima media thickness when compared to placebo, but calcium channel blockers seem more effective than other antihypertensive drugs when studied in an active-control setting.[60]

Antihypertensive drugs in general have been shown to reduce the incidence of cardiovascular disease and mortality, although potential differences between the various antihypertensive agents remain controversial.[61] In general, meta-regression analysis have shown that the blood-pressure lowering effect rather than any pleiotropic drug properties explained most of the cardiovascular protection conferred by antihypertensive drugs, but also that calcium channel blockers, independent of their blood pressure lowering activity, might have a small additional beneficial effect in the prevention of stroke.[62] This, in turn, may be mediated by the beneficial effects on carotid intima media thickness, or by favourable effects on fibrinolysis.

In short, there is convincing evidence that antihypertensive drugs reduce the incidence of cardiovascular disease and mortality. There is evidence that both ACE inhibitors and calcium channel blockers improve fibrinolysis, partly independently of their blood pressure lowering effect. In addition, improving blood pressure might have a beneficial influence on other markers of endothelial function as well. Extensive evidence exists that all antihypertensive agents reduce carotid intima media thickness, but that calcium channel blockers might do so in a (partly) blood pressure independent way.

Statins

Statins, or HMG-CoA reductase inhibitors, are used to lower cholesterol levels and are frequently prescribed in diabetic and non-diabetic patients. Although cholesterol lowering is clearly linked to decreased atherosclerosis and a lower incidence of cardiovascular disease in hypercholesterolemic subjects, several recent statin trials in subjects with low or normal cholesterol levels, have suggested additional benefits beyond cholesterol lowering.[63-66] The mechanism through which these suggested pleiotropic effects of statins take place, are still largely unclear. Several studies have shown contradictory results on the ability of statins to reduce levels of CRP. However, meta-analyses of these trials show statins reduce CRP levels by about 30%.[67, 68] These meta-analyses also showed this CRP-lowering effect to be largely (~90%) dependent on the LDL-lowering effect. Some studies found statins to reduce levels of PAI-1,[69-71] while others did not.[72, 73] One study showed no effects on sICAM-1, sVCAM-1, or vWf.[69] An improvement in FMD which was independent of the lipid-lowering effect was shown in one study.[65]

Large intervention trials and subsequent meta-analysis have shown statins to reduce the risk of vascular events and all-cause mortality.[74, 75]

In short, convincing evidence shows that statins reduce cardiovascular morbidity and mortality. There is some evidence that this might be, at least in part, independent of the lipid-lowering effect of the drug. Which mechanisms might explain the favourable effects on cardiovascular disease remain largely unknown. A relatively large reduction in CRP-levels might contribute, although this reduction is probably largely LDL-dependent. Alternatively, improvements in fibrinolysis (PAI-1) and potential influences on NO synthesis promoting vasodilatation are other proposed mechanisms that may play a role.[65]

Methodological considerations and critical remarks

The Hoorn Study (Chapter 2)

The Hoorn Study is a population-based cohort study of glucose tolerance and cardiovascular disease in a Caucasian population in Hoorn, the Netherlands, of which the baseline measurements were performed from October 1989 to February 1992.[76, 77] Briefly, a random sample of all men and women aged 50-75 was drawn from the municipal population registration office of Hoorn; 2484 individuals participated (response rate 71%). The data used in this thesis (Chapter 2) resulted from an age-, sex-, and glucose-tolerance-stratified random subsample (n=631; response rate 89%), in whom an extensive investigation of diabetes complications was performed.[76, 77]

Strengths of this study include its population-based design; the long follow-up (up to 13 years); the limited loss to follow-up; the extensive characterization of participants at baseline; and the high agreement reached between coders on the classification of cardiovascular mortality (92.3%).

However, there are some limitations. We studied only Caucasian individuals and the results therefore are not necessarily valid for other ethnicities. Secondly, the incomplete assessment of endothelial function and inflammatory activity may have increased non-differential misclassification, leading to an underestimation of the hazard ratios presented here. However, our results were robust and consistent with previous experience. Finally, because traditional risk factors were measured only once, we may have underestimated their associations with mortality to some extent, although previous analyses from the Hoorn Study have shown that traditional risk factors, even if measured only once, do in fact predict mortality.[78]

The HOME trial (Chapters 3 – 6)

The HOME trial included 390 patients with type 2 diabetes mellitus between 30 and 80 years of age who had received a diagnosis of diabetes after the age of 25, who had never had an episode of ketoacidosis, and whose blood-glucose-lowering treatment had previously consisted of oral agents but now only consisted of either insulin (n=345) or insulin and metformin (n=45). We excluded pregnant women and women trying to become pregnant, patients with a Cockcroft-Gault-estimated creatinine clearance < 50 ml/min per 1.73m2 or low plasma cholinesterase (reference value < 3.5 units/l) as a marker of liver failure. Patients with congestive heart failure (New York Heart Association class III/IV) or other serious medical or psychiatric disease were excluded as well. The HOME trial was conducted in the outpatient clinics of three non-academic hospitals (Hoogeveen, Meppel, and Coevorden; The Netherlands). Patients were randomly allocated to either placebo or metformin by aid of a computer program, which allocated a random number to identical looking boxes of either metformin or placebo. The trial design consisted of three phases: the 12 week pre-randomisation phase, in which patients were treated with insulin only, and concomitant medication was discontinued; the 16 week short-term treatment phase, at the beginning of which patients were randomised to receive either metformin or placebo in addition to insulin therapy; and the 48 month long-term treatment phase. After the short-term treatment phase an interim analysis took place, during which the treatment codes were not disclosed to the investigators, which was followed by the long-term treatment phase, a continuation of the short-term treatment phase.

Strengths of our study include its randomised, placebo-controlled, double-blind design, its long follow-up period of 4.3 years with frequent clinical visits and serum collection, and finally the sustained participation of patients in the trial after the occurrence of a nonfatal disease-related end point, thereby reducing dropout bias.

However, there are some limitations. First, its relatively small sample size and consequently limited power may have obscured smaller treatment effects. To increase the power of our study, disease-related end points were constructed by combining separate clinical events regarding microvascular and macrovascular disease. An assumption in the construction of these disease-related end points is that its components are equally important, which is not necessarily true. In addition, the hypothesis that metformin influences both microvascular and macrovascular disease through shared underlying pathophysiologic characteristics, in the way, for instance, that

obesity does, may not be correct. Second, there was an imbalance between the two treatment groups after randomisation. We adjusted for unbalanced variables in all analyses, but we cannot rule out some residual confounding. To adjust for the difference in prior cardiovascular disease, we constructed a way of measuring cardiovascular history, which might not optimally reflect the medical history and severity of cardiovascular disease at baseline. Therefore, the results, especially with regard to the secondary, macrovascular end point, must be interpreted with caution. Third, the results on low-grade inflammation must be interpreted with caution. We did not include other markers of inflammation, such as interleukin 6, tumour necrosis factor alpha and serum amyloid A protein. Low-grade inflammation was estimated by two markers, i.e. CRP and sICAM-1, whereas endothelial dysfunction was represented by six markers. In addition, sICAM-1 can be regarded as a marker of both endothelial function as inflammation.[79, 80] This may have led to an underestimation of the effect of metformin on low-grade inflammation. Fourth, we measured only total vitamin B12 levels and did not measure possibly more precise indicators of vitamin B12 status such as holotranscobalamine-II or methylmalonic acid. In addition, we may have somewhat underestimated the adverse effects of metformin, because all participants in our trial received frequent dietary counselling, which may have attenuated the impact of metformin treatment on vitamin status and which may not be available in routine clinical practice. Fifth, although all patients were treated in non-academic hospitals, they did receive more intensive care than normally available in such centres, and our results may therefore not be generalisable to patients in other settings.

Summary, practical implications, and future research

Summary

Markers of endothelial dysfunction and inflammation were associated with higher risks of cardiovascular mortality, especially in type 2 diabetes. These associations were independent of other cardiovascular risk factors, and also appeared mutually independent, indicating that they may represent largely distinct pathways of disease and therefore distinct targets for intervention. Low grade inflammation explained about 25% and endothelial dysfunction about 34% of the increased mortality risks associated with type 2 diabetes. Long-term treatment with metformin improved body weight and insulin requirements, but did not decrease the risk of the primary end point, which consisted of both microvascular and macrovascular disease. However, it did decrease the risk of the secondary, macrovascular end point, which was partly (about 40%) related to the prevention of weight gain, but not to the decrease in insulin levels due to metformin treatment. Treatment with metformin improved endothelial dysfunction, which explained 35% of the reduction in the risk of macrovascular morbidity and mortality associated with metformin treatment. Treatment with metformin reduced levels of vitamin B12, increasing the chance of developing vitamin B12 deficiency. This did not result in significantly higher levels of homocysteine in the metformin group. However,

significantly increased homocysteine levels were found in individuals with clinical B12 deficiency, irrespective of treatment.

Practical implications

In general practice, when, owing to the progressive nature of type 2 diabetes, insulin treatment is required, patients may benefit greatly if metformin treatment is continued. However, current guidelines make no recommendations on the detection and prevention of vitamin B12 deficiency during metformin treatment. Our data provide a strong case for routine assessment of vitamin B12 levels during long-term treatment with metformin.

Future research

Additional, long-term trials on the effect of metformin on cardiovascular outcome are needed before unambiguous conclusions can be drawn. In addition, the Hoorn study showed low-grade inflammation to be an important predictor of cardiovascular morbidity and mortality. However, inflammation has remained somewhat less intensively investigated in the HOME trial than endothelial dysfunction. Future research might focus on the effects of metformin on other markers of inflammation, such as interleukin 6, tumour necrosis factor alpha, and serum amyloid A protein. Improvement of endothelial function seems an important mechanism by which metformin may reduce cardiovascular morbidity and mortality. However, it is still unknown exactly how metformin improves endothelial function. Alternatively or additionally, metformin may improve endothelial function by decreasing advanced glycation endproduct levels, by altering the secretion of adipocyte-derived mediators (such as free fatty acids, leptin, resistin, and adiponectin), and (or) by improving insulin sensitivity, of which a change in insulin dose may be an insufficiently accurate marker. These possibilities require further study.

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Chapter 8

Nederlandse samenvatting
Dankwoord
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Nederlandse samenvatting

Historisch perspectief van de HOME studie

In 1998 suggereerden de eerste resultaten van de UKPDS,[1] een belangrijk onderzoek naar de invloed van glycemische instelling op het ontstaan van complicaties bij diabetes, een cardioprotectieve invloed van metformine, die onafhankelijk leek van de mate van glycemische instelling. Echter, het onderzoeksontwerp en de statistische analysen van de UKPDS gaven aanleiding tot een aanzienlijk debat. Bovendien werden de mechanismen waarop metformine cardiovasculaire ziekte zou beïnvloeden niet opgehelderd. Deze vragen waren de aanleiding voor het ontwerp van het gerandomiseerde, placebo-gecontroleerde, multicenter studie "Hyperinsulinaemia: the Outcome of its Metabolic Effects" (HOME), waarin bovenstaande kon worden onderzocht.

In de jaren '90 toen het studieprotocol voor de HOME studie opgesteld werd, was het onderzoeksontwerp erop gericht te onderzoeken of metformine de incidentie van micro- and macrovasculair ziekte bij type 2 diabetes kon verlagen, en zoja, of een verlaging van de insulinespiegels hieraan ten grondslag zou liggen. Metformine, een medicijn dat al lang gebruikt wordt en veilig is gebleken en dat de insulinegevoeligheid doet toenemen, werd gebruikt tegenover een placebo, naast gelijktijdig gebruik van insuline, om zo de hyperinsulinemie in de metformine groep te verlagen, met de opzet de glycemische instelling tussen beide groepen vergelijkbaar te houden. Recenter wordt ook meer gekeken naar andere risicofactoren dan hyperinsulinemie als mogelijke kandidaten die het eventuele voordelige effect van metformine zouden kunnen verklaren.

Er zijn maar weinig nadelen aan het gebruik van metformine. Metformine induceert echter vitamine B12 malabsorptie, hetgeen kan leiden tot een verhoogd risico op het ontwikkelen van een vitamine B12 deficiëntie,[2-4] een klinisch belangrijke en makkelijk behandelbare aandoening. Bovendien is uit ander onderzoek gebleken dat het gebruik van metformine geassocieerd is met verlaagde folaat concentraties; het mechanisme dat hieraan ten grondslag zou liggen is echter niet aangetoond.[5] Tot slot kunnen de verlagingen in folaat en vitamine B12 leiden tot een toename in de homocysteine concentraties. Homocysteine is een onafhankelijke risicofactor gebleken voor het optreden van cardiovasculaire ziekte, in het bijzonder bij patiënten met type 2 diabetes.[6-8]

Huidige bevindingen

De associatie tussen endotheeldysfunctie en ontsteking en cardiovasculaire ziekte (Hoorn studie)

Endotheeldysfunctie en laaggradige ontsteking zijn geassocieerd met een groter risico op cardiovasculaire mortaliteit. In het geval van endotheeldysfunctie was deze

associatie sterker bij individuen met diabetes dan bij niet-diabeten. Voor zowel endotheeldysfunctie als laaggradige ontsteking was de associatie met cardiovasculaire mortaliteit onafhankelijk van andere cardiovasculaire risicofactoren, alsook onafhankelijk van elkaar, wat suggereert dat ze grotendeels aparte ziektemechanismen vertegenwoordigen en dus ook verschillende aangrijppunten voor interventie zouden vormen. Laaggradige ontsteking verklaarde ongeveer 25%, endotheeldysfunctie ongeveer 34%, en samen ongeveer 43% van het verhoogde risico op sterfte die gepaard gaat met type 2 diabetes.

Korte termijn effecten van metformine op endotheeldysfunctie en ontsteking (HOME studie)

Korte termijn behandeling (16 weken) met metformine was geassocieerd met afnames in plasma concentraties van vWf, sVCAM-1, sE-selectin, t-PA en PAI-1. Veranderingen in de plasma concentraties van deze markers waren onafhankelijk van de door metformine geïnduceerde verbeteringen in gewicht, glycemische instelling, insulinedosering en lipidenprofiel. De enige uitzondering was de afname in plasma sE-selectin, welke gedeeltelijk verklaard werd door de met metformine geassocieerde verbetering in glycemische instelling en triglyceride concentraties. We vonden geen effect van korte termijn behandeling met metformine op markers van laaggradige inflammatie, sICAM-1 en CRP.

Lange termijn effecten van metformine op het metabolisme en micro- en macrovasculaire ziekte (HOME studie)

Lange termijn behandeling (4.3 jaren) met metformine verbeterde het gewicht, de insulinebehoefte en had een bescheiden gunstig effect op de glycemische instelling (ondanks het streven naar een vergelijkbare glycemische instelling in beide groepen), maar had geen effect op bloeddruk of het lipidenprofiel. Behandeling met metformine verminderde het risico op het primaire eindpunt niet, welke bestond uit zowel microvasculaire als macrovasculaire ziekte. Echter, behandeling met metformine verlaagde wel het risico op het secondaire, macrovasculaire eindpunt (hazard ratio 0.61; 95% CI, 0.40-0.94; P=0.02), hetgeen gedeeltelijk (ongeveer 40%) kon worden verklaard door het voorkómen van gewichtstoename gedurende de metformine behandeling. Alle andere met metformin geassocieerde veranderingen in metabole of hemodynamische variabelen, waaronder hyperinsulinemie, droegen niet bij aan het gunstige effect van metformine op macrovasculaire ziekte. Er werd geen verbetering in microvasculaire ziekte gevonden.

Lange termijn effecten van metformine op endotheeldysfunctie en inflammatie en de associatie met cardiovasculaire ziekte (HOME studie)

Lange termijn behandeling (4.3 jaren) met metformine verlaagde concentraties van vWf, sVCAM-1, t-PA, PAI-1, en sICAM-1, welke, behalve in het geval van sICAM-1, gedeeltelijk (ongeveer 60%) onafhankelijk waren van de met metformin geassocieerde veranderingen in HbA_{1c}, insuline dosering en gewicht. De verlaging in sICAM-1

concentraties werd geheel gemedieerd door metformin geïnduceerde verbeteringen in glycemische instelling en lagere insuline niveaus. Er werd geen effect van metformine gevonden op urine albumine uitscheiding of CRP. Van alle markers bleken alleen vWf en sVCAM-1 voorspellers van macrovasculaire ziekte; verbeteringen in vWf and sVCAM-1 konden 35% verklaren van de verlaging van het risico op macrovasculaire morbiditeit en mortaliteit geassocieerd met de behandeling met metformine.

Lange termijn effecten van metformine op vitamine B12, folaat en homocysteine concentraties (HOME studie)

Lange termijn behandeling (4.3 jaren) met metformine verlaagde concentraties van vitamine B12; de hazard ratio voor het ontwikkelen van vitamine B12 concentraties onder 150 pmol/l vergeleken met placebo was 5.5 (95% CI: 1.6 tot 19.1). Er werd een kleine, maar significante afname in folaat concentraties in de metformine groep gevonden, hetgeen niet langer statistisch significant was na correctie voor verschillen tussen beide groepen in BMI en roken. De afname in vitamine B12 concentraties was geassocieerd met een toename in homocysteine concentraties, welke niet significant verschilde tussen de twee behandelgroepen. Onafhankelijk van de behandeling namen de homocysteine concentraties significant toe in individuen bij wie de vitamine B12 concentraties onder het niveau kwamen, dat vaak wordt beschouwd als een klinisch relevante deficiëntie, dat wil zeggen onder 150 pmol/L.

Samenvatting, praktische implicaties en toekomstig onderzoek

Samenvatting

Markers van endotheeldysfunctie en inflammatie waren geassocieerd met een hoger risico op cardiovasculaire sterfte, vooral in het geval van type 2 diabetes. Deze associaties waren onafhankelijk van andere cardiovasculaire risicofactoren, alsook onafhankelijk van elkaar, wat suggereert dat ze grotendeels aparte ziektemechanismen vertegenwoordigen en dus ook verschillende aangrijppunten voor interventie zouden vormen. Laaggradige inflammatie verklaarde ongeveer 25% en endotheeldysfunctie ongeveer 34% van het verhoogde risico op sterfte die gepaard gaat met type 2 diabetes. Lange termijn behandeling met metformine verlaagde het gewicht en de insulinebehoefte, maar verlaagde het risico op het primaire eindpunt niet, welke bestond uit zowel micro- als macrovasculaire ziekte. Echter, behandeling met metformine verlaagde wel het risico op het secondaire, macrovasculaire eindpunt, hetgeen gedeeltelijk (ongeveer 40%) kon worden verklaard door het voorkómen van gewichtstoename, maar niet door lagere insulinespiegels, gedurende de metformine behandeling. Behandeling met metformine verbeterde de endotheeldysfunctie, wat vervolgens 35% van het lagere risico op macrovasculaire morbiditeit en mortaliteit gepaard gaande met het gebruik van metformine kon verklaren. Behandeling met metformine verlaagde de vitamine B12 concentraties en verhoogde de kans op het ontwikkelen van een vitamine B12 deficiëntie. Dit resulteerde niet in significant hogere concentraties homocysteine in de metformine groep. Echter, in individuen met een

vitamine B12 deficiëntie werden significant hogere homocysteine concentraties gevonden, onafhankelijk van de behandeling.

Praktische implicaties

In de algemene praktijk kunnen patiënten voordeel opdoen wanneer de behandeling met metformine wordt voorgezet, op het moment dat, door het progressieve karakter van type 2 diabetes, het noodzakelijk is geworden met insuline te behandelen. De huidige richtlijnen doen echter geen aanbevelingen over het voorkomen en detecteren van vitamine B12 deficiënties tijdens de behandeling met metformine. Onze resultaten bieden sterke argumenten voor het routinematig bepalen van vitamine B12 niveaus tijdens een langdurige behandeling met metformine.

Toekomstig onderzoek

Er zijn aanvullende lange termijn onderzoeken nodig over de effecten van metformine op cardiovasculaire uitkomsten voordat hierover eenduidige conclusies kunnen worden getrokken. De Hoorn studie liet zien dat laaggradige inflammatie een belangrijke voorspeller van cardiovasculaire morbiditeit en mortaliteit is. Echter, inflammatie is in de HOME studie minder intensief onderzocht dan endotheeldysfunctie. Toekomstig onderzoek zou zich kunnen richten op de effecten van metformine op andere markers van inflammatie, zoals interleukine 6, tumor necrosis factor alpha en serum amyloid A proteïne. Verbetering van endotheelfunctie blijkt een belangrijk mechanisme waardoor metformine de cardiovasculaire morbiditeit en mortaliteit verlagen kan. Hoe metformine endotheelfunctie precies verbetert, is echter nog steeds onbekend. Metformine zou dit kunnen bewerkstelligen door `advanced glycation endproducts` te verlagen, door de secretie van mediatoren van adipocyten afkomstig (zoals vrije vetzuren, leptine, resistine en adiponectine) te verlagen, en (of) door de insulinegevoeligheid te verbeteren, waarvan een verandering in insulinedosering een onvoldoende accurate marker kan zijn. Toekomstig onderzoek kan zich op deze kwesties richten.

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Curriculum Vitae

Jolien de Jager was born in 1978, in Groningen, the Netherlands. She spent her early childhood in a small village in the most northern part of Groningen, Termunten (pop. 420). Later, she and her family moved to Zutphen where she graduated from secondary school in 1996. While she waited to be accepted to study medicine, she studied psychology for two years at the University of Groningen. In 1998, she started her medical study at that same university. In 2002 she obtained her Master in Science degree, after studying the associations between repeatlenghts and age of onset of autosomal dominant cerebellar ataxia at the department of Clinical Genetics. Later in 2002, she postponed her internship to join the HOME study group for two years, where she did clinical work and collected data for her thesis. In 2004 she resumed her internship, to obtain her medical degree with honor in 2006. After finishing medicine, she worked as a resident in psychiatry in the University hospital of Groningen for almost a year, and as a resident in the intensive care unit of thoracic surgery for another year. In 2008 she started her residency in ophthalmology at the Academic Medical Centre of Amsterdam, and expects to finish in 2013. She now lives in Amsterdam, with her husband and son.